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(54) Title: **METHODS FOR QUANTITATIVE DETERMINATION OF B-FIBRONECTIN IN BIOLOGICAL FLUIDS AND TISSUES**

(57) Abstract: Quantitative determination of fibronectin isoform B (B-FN) and other insoluble marker proteins, including quantitative determination of B-FN in plasma and other biological fluids, employing gelatin that binds B-FN, also quantitative determination of insoluble marker proteins such as B-FN in tissue. Provision of quantitative information on angiogenesis through detection of B-FN as an indicator of malignancy in neoplasia.

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METHODS FOR QUANTITATIVE DETERMINATION OF B-FIBRONECTIN
IN BIOLOGICAL FLUIDS AND TISSUES

The present invention relates in various aspects to
5 quantitative determination of fibronectin isoform B (B-FN).
More specifically, aspects of the invention relate to
quantitative determination of B-FN in plasma and other
biological fluids, employing antibody specific for the ED-B
domain unique to B-FN. In other aspects, the present
10 invention relates to quantitative determination of insoluble
marker proteins such as B-FN in tissue. In still further
aspects the invention relates to provision of quantitative
information on angiogenesis through detection of B-FN as an
indicator of malignancy in neoplasia.

15 The quantitative aspects of the present invention provide
prognostic information of value in a clinical setting, and
numerous advantages over prior art detection of B-FN.

20 Early stage detection is of critical importance in the
diagnosis and treatment of patients with angiogenesis related
disorders such as cancer. This detection is aided by the use
of markers produced by pathological tissues but absent from
normal tissue. The development of quantitative immunoassays
25 for the detection of such markers has allowed for the
screening and diagnosis of (potential) cancer patients. Here
we describe the development of three diagnostic methods which
utilize the detection of a tumor and angiogenesis associated
Fibronectin isoform called B-FN for the detection of
30 neoplastic disease and the evaluation of tumor malignancy.

The Fibronectin (FN) isoform containing the Extra Domain B (B-FN) is a tumor-associated protein which is undetectable in

normal tissue but is associated with the pronounced remodeling of the extracellular matrix during tumor growth and the formation of new blood vessels (angiogenesis) (Carnemolla et al. 1989; Castellani et al. 1994; Kaczmarek et al. 1994). B-FN is generated by the inclusion of the Extra Domain B (ED-B) during alternative splicing of FN's primary transcript (Figure 1) (Carnemolla et al. 1989; Castellani et al. 1986; Zardi et al. 1987). The use of human recombinant antibodies against this highly conserved domain has allowed for extensive characterization of B-FN as a tumor and angiogenesis marker (Carnemolla et al. 1996; Neri et al. 1997). Furthermore, in addition to functioning as a marker, the incorporation of B-FN into the extracellular matrix of tumor tissue and newly formed vessels provides an antigen for tumor and angiogenesis targeting (Carnemolla et al. 1996; Neri et al. 1997; Tarli et al. 1999).

However, prior to the making of the present invention, assays used to detect B-FN have not allowed for quantitative determination. The present inventors have developed quantitative assays and have further shown that quantities of B-FN in plasma, in solid tissue and in angiogenic blood vessels, can be used in prognostic contexts, i.e. in characterising the nature and severity of a tumor and of tumor progression and metastases in a clinical setting. This is of great value to clinicians, allowing for appropriate therapeutic intervention and for monitoring of success or failure of treatment.

Brief Description of the Figures

Figure 1 shows a schematic domain structure of FN, including location of type I, II and III domains in FN with their

corresponding functional interactions. In addition the locations of the alternatively spliced domains ED-A, ED-B and IIIICS are indicated. Arrows demonstrate the approximate binding of antibodies against total FN (human specific, IST-4), A-FN (various species, IST-9), and B-FN (pan-species, human recombinant anti-ED-B).

Figure 2 shows standard curves utilized in the quantitative detection of FN. Purified plasma and Wi38va13 FN were utilized as standards for the quantitation of FN in unknown samples. The sensitivity of A-FN and B-FN detection after immobilization of Wi38va13 FN to gelatin was 2.5 ng/ml and 1 ng/ml respectively. The detection of total FN achieved after the immobilization of plasma FN was 0.3 ng/ml. All three standards curves can be fit to a 4-parameter plot and span an approximate target range of 100 ng/ml.

Figure 3 shows results of quantitative detection of FN including the A and B-FN isoforms. The levels of FN in plasma from a population of control individuals were analyzed for using the gelatin immobilization ELISA. The data from each individual are presented as a circle on this scatterplot. Horizontal bars within each data set represent the mean.

Figure 4 shows results for detection of tumor derived FN in plasma from tumor bearing mice. The elevation of FN levels in plasma from tumor bearing mice was determined using the gelatin immobilization ELISA as described in materials and methods. Each time point represents the average of three animals. Figure 4A shows the levels of B-FN and total FN determined in BALB/c bearing the murine adenocarcinoma C51. Figure 4B shows the specific elevation of B-FN in BALB/c bearing C51 analyzed by calculating the % B-FN.

Figure 5 shows a scatterplot of B-FN detection in plasma from control individuals and tumor patients represented as % of total FN. Plasma from control individuals and tumor patients was analyzed using gelatin immobilization ELISA as described in materials and methods. The 97.5% cutoff line is indicated at 0.56% and the mean of each data set is represented as a horizontal bar.

Figure 6 shows results of quantitative detection of insoluble FN in homogenized tissue.

Figure 6A shows a standard curve plotted based on the CPM bound to known quantities of FN-sepharose.

Figure 6B shows the CPM retained within the insoluble component of the homogenate from a tumor (F9) and a control sample (liver). The same experiment was performed with anti-ED-B antibody pre-incubated with recombinant ED-B to demonstrate specific binding (F9 + ED-B and liver + ED-B).

Figure 7 shows quantitative detection of insoluble B-FN and total FN in human tumors and normal tissue.

Normal tissue and tumor tissue was processed and analyzed as described in materials and methods. The quantity of B-FN and total FN was determined in lung tissue and intra cranial tissue.

Figure 7A shows results for lung. Figure 7B shows results for brain.

Figure 8 shows results for determination of the vascular density, the angiogenic index, and the number of proliferating cells in cryosections from high and low grade Astrocytoma.

Cryostat sections of high and low grade Astrocytoma were prepared as described in materials and methods.

Figure 8A shows the vascular density, determined as the total number of blood vessels.

5 Figure 8B shows the angiogenic index, determined as the percentage of vessels which are B-FN+.

Figure 8C shows the number of proliferating cells, determined through the detection of KI67 positive cells.

Every circle represents the mean data from five separate high magnification fields (HMF) for one individual.

The inventors have developed a quantitative assay specific for the ED-B domain of B-FN that is useful in determining B-FN presence in biological fluids such as plasma, cerebral-spinal fluid and cystic fluid.

Conventional quantitative methods for soluble tumor markers are based on direct immobilization of the antigen by an antigen-specific antibody.

20

Previously, Claudepierre et al. (1999) employed an assay for B-FN in plasma in which they used an antibody that binds B-FN at a site outside of ED-B. The assay was not quantitative.

The present inventors realised that as ED-B is unique to B-FN it is likely to be the best target for an assay for quantitative determination of the presence of B-FN in any sample. When this was attempted, however, the inventors found that ED-B in plasma B-FN is cryptic and cannot be bound by anti-ED-B antibodies. The inventors suspect that the ED-B in plasma B-FN is inaccessible due to cryptic folding of plasma B-FN or specific binding of a plasma component to B-FN.

Having tried various approaches, the inventors finally solved the problem by immobilising plasma FN in the assay by means of gelatin. This they surprisingly found results in unmasking of ED-B allowing for subsequent quantitative detection of B-FN in a gelatin immobilization Enzyme Linked Immunosorbent Assay (ELISA). Soluble FN is immobilized in microtiter plates through binding to gelatin coated wells. Gelatin is known to bind FN (Damas et al. 1986; Engvall and Ruoslahti 1977; Gao and Groves 1998; Hynes 1990), but its use in a quantitative assay in accordance with the present invention, relying on the surprising ability to unmask the cryptic ED-B, has not been suggested.

The present invention thus generally provides an assay method for quantitatively determining the presence of B-FN in a body fluid taken or collected from an individual. The experimentation below shows that an elevated level of B-FN in a body fluid such as plasma, cerebral-spinal fluid or cystic fluid is indicative of the presence of a tumor in the individual from which the fluid sample was taken. In embodiments of the present invention, a solid support is employed for capture of B-FN from a body fluid passed over the support, the support having attached to it gelatin, able to bind fibronectin. Thus, a gelatin-coated plate or beads may be employed.

A further aspect of the present invention provides a kit for use in an assay method of the invention. Such a kit may in a preferred embodiment comprise a gelatin-coated solid support, such as a plate or bead, and a specific binding member for B-FN e.g. an antibody molecule, especially an antibody molecule specific for ED-B of B-FN

According to a first aspect of the present invention there is provided a method of determining the presence of fibronectin isoform B (B-FN) in a body fluid taken or collected from an individual, the method comprising:

5 passing the body fluid over a solid support to which is attached gelatin, able to bind fibronectin, whereby fibronectin, if present in the body fluid, is bound by the gelatin and retained on the solid support;

10 determining the presence or absence of B-FN retained on the solid support, by determining ability of a specific binding member for the Extra Domain B (ED-B) of B-FN to be retained on the solid support.

The specific binding member is generally an antibody molecule.

15

Binding of fibronectin to gelatin on a solid support is demonstrated herein to unmask the cryptic ED-B domain unique to fibronectin isoform B, allowing for determination of the presence of B-FN.

20

Preferably, the determination is quantitative, the amount of specific binding member retained on the solid support being determined and, if necessary, compared with appropriate calibrated standards or control samples containing known
25 amounts of B-FN.

In addition, the presence of other isoforms of fibronectin, such as A-FN, may be determined and may be quantitated.

30 The term "antibody molecule" may be used herein (for any specific binding member as discussed) to refer to a complete antibody or any antibody fragment, whether natural or synthetic, that is able to bind the relevant antigen, here

fibronectin, especially B-FN, in particular ED-B of B-FN. Examples of binding fragments include the well-known scFv, Fv, Fab, Fd (VH and CH1) and dAb (VH domain) molecules. Any suitable antibody fragment can be linked to a peptide or protein domain or enzyme, e.g. as a fusion protein, or to a label.

The specific binding member, e.g. antibody molecule, employed to determine the presence of fibronectin retained on the solid support may be labelled with a radioisotope such as ^{32}P , ^{35}S , ^3H , or ^{125}I , an enzyme such as horse radish peroxidase, or fluorescence such as FITC or rhodamine. In some embodiments, a further specific binding member directed against the second specific binding member is employed, where the further specific binding member is labelled, e.g. with horseradish peroxidase or other detectable enzyme. Where for example the specific binding member is a murine monoclonal antibody against fibronectin, the further specific binding member may be a rabbit-anti-mouse antibody molecule. Variations and alternatives are apparent and available to those skilled in the art.

The amount of fibronectin (e.g. B-FN) retained on the solid support may be quantitated by means of a label or labelling system (e.g. horseradish peroxidase).

The body fluid sampled may be for example plasma, cerebral-spinal fluids, cystic fluid, lymph node aspirations, ascites fluid, urine or seminal fluid.

A further aspect of the present invention provides a kit for use in an assay method of the invention. A kit according to the invention may comprise (i) a solid support to which is

bound gelatin able to bind fibronectin, and (ii) a specific binding member for ED-B of B-FN. The kit may also comprise one or more additional components useful in performance of an assay in accordance with the invention, for instance one or more of the following: solutions, a specific plate, binding buffer, coating buffer, washing buffer, a label or labelling system, a developing system including a radio-chemical, photo-chemical, enzyme, biochemical or chemical reaction system.

10 Results obtained using this quantitative detection of gelatin immobilized FN are included in Examples 1, 2 and 3 below. These show:

1 FN, including the isoforms A-FN and B-FN can be
15 quantitatively detected in plasma from mice and humans after immobilization onto gelatin. An analysis of a population of control individuals demonstrates that the plasma levels of B-FN are lower and with less variability than the level of A-FN.

20 2 Tumor bearing mice acquire elevated levels of B-FN in their plasma, while control mice maintain a low level of plasma B-FN.

3 An analysis of a cancer patient population demonstrates a
25 significant elevation of B-FN in 46% of all individuals. This elevation is more frequent and more specific than the elevation of A-FN.

4 The gelatin immobilization ELISA can also be used to
30 quantitatively detect total FN and the alternatively spliced variants in cerebral-spinal fluid and cystic fluid.

Because this assay utilizes immobilization of FN to gelatin, all FN isoforms can be readily detected without significant modification of the assay. This greatly increases the amount of information regarding circulating tumor associated FN that
5 can be obtained from a single sample.

Further problems have been encountered in the prior art for determination of B-FN presence in tissues.

10 Immunohistochemical analysis and immuno-targeting of B-FN in mouse tumor models has provided significant insight into the distribution and targeting potential of this protein (Neri et al. 1997; Tarli et al. 1999). Immunohistochemical data demonstrates that B-FN can be found associated with the tumor
15 stroma, the tumor vessels and the tumor cells themselves (Berndt et al. 1998; Castellani et al. 1995; Kosmehl et al. 1999). Furthermore, substantial data in tumor/angiogenesis targeting and therapy by means of anti-ED-B antibodies has been accumulated in mouse tumor models (Carnemolla et al.
20 1996; Neri et al. 1997; Tarli et al. 1999). However, quantitative analysis of B-FN and other tumor markers that are insoluble components of the tumor tissue has not been achieved because of the heterogeneity of the tissue and the variability in antigen density and distribution. A quantitative assessment
25 of insoluble B-FN would assist in evaluating the diagnostic and targeting potential of B-FN in human tumors.

The present inventors have developed methodology for quantitative determination of insoluble B-FN in tissues. In
30 their work they have determined the quantity of antigen available for tumor diagnostics and targeting, and determined diagnostic and prognostic value of quantitative B-FN detection by correlating quantity of B-FN with the grade of tumor.

In accordance with the present invention insoluble B-FN (if present) within a tissue homogenate is bound by labelled anti-B-FN antibody (e.g. radiolabelled, such as with I^{125} detectable with a gamma counter). Unbound antibody is separated from the bound material (e.g. by means of vacuum filtration) and remaining label is determined.

Using this methodology the inventors have found that B-FN is readily and quantitatively detected in the tumor tissue from both experimental mouse tumor models as well as human tumors. In contrast, B-FN is undetectable in normal tissues.

The use of this aspect of the present invention and results so obtained are described in Examples 4 and 5 below.

According to a further aspect of the present invention there is provided a method of quantitating the amount of an insoluble marker protein in a sample of tissue or tumor, the method comprising:

homogenising a tissue or tumor sample to provide a homogenate;

contacting the homogenate with a specific binding member for the insoluble marker protein and incubating under conditions wherein the specific binding member binds the insoluble marker protein if present in the homogenate;

separating insoluble material from soluble material in the homogenate, the soluble material including specific binding member unbound to the insoluble marker protein;

determining the amount of specific binding member in the insoluble material, correlating with the amount of insoluble marker protein in said sample.

The amount of insoluble marker protein may be indicative of the state or severity of a lesion or disorder in the tissue, e.g. the grade or severity of neoplasia. The amount may be correlated with known states, grades or severity to provide
5 diagnostic and/or prognostic information.

Generally the specific binding member is an antibody molecule, and may be any antibody molecule as discussed already above in connection with other aspects of the invention. Similarly,
10 one or more labels or labelling systems may be employed as have already been discussed.

Correlation of the amount of specific binding member or label with amount of insoluble material may involve comparison with
15 an appropriate calibrated standard or control. In a preferred embodiment, a controlled amount of insoluble marker protein immobilization to Sepharose beads or other solid supports, such as gelatin coated beads or Nitrocellulose, is employed as a control. The amount of insoluble marker protein in the test
20 sample may be calculated with reference to the known amount in the control.

In preferred embodiments, the insoluble marker protein is fibronectin, especially B-FN, in which case the specific
25 binding member is preferably specific for ED-B. Other insoluble marker proteins the presence of which in a tissue sample or tumor may be quantitatively determined in accordance with embodiments of the present invention include A-FN, the tumor specific splice variants of Tenascin, and tumor specific
30 splice variants of Laminin.

As noted, B-FN is, with very rare exceptions, virtually undetectable in normal adult tissues. However, B-FN is notably

upregulated in foetal and neoplastic tissues (Carnemolla et al. 1989). Furthermore, B-FN accumulates around neovasculature during angiogenic processes but not around mature vessels thus providing a marker for angiogenesis (Castellani et al. 1994). The vascular density in various tumors has been correlated with the malignancy of various kind of tumors and, in turn, with the prognosis. Nevertheless there are conflicting reports as to whether microvessel density is associated with cancer malignancy and prognosis. These conflicting results can be due to the fact that vascular density does not give information on the level of angiogenic activities taking place within a tissue since vascular density has been evaluated so far using pan-endothelial markers such as factor VIII (Toi et al. 1995; Weidner 1993).

Further work of the present inventors has established a correlation between the proportion (e.g. percentage) of blood vessels in a tissue that contain B-FN compared with the total number of blood vessels and grade or severity of neoplasia. Thus, further aspects of the invention relate to provision of an angiogenic index useful as an indicator of malignancy in neoplasia.

The present inventors have devised an approach using a double staining procedure to evaluate the percentage of vessels undergoing angiogenic processes can be considered as an angiogenic index (AI) of a tissue. Experimental support for and use of this aspect of the present invention is included in Example 6 below. Using this procedure, the inventors have evaluated the AI in samples of low and high grade astrocytomas and compared the values with vascular density and the proliferative activity within the tissue as evaluated using

the an antibody against the protein KI67 which is expressed in proliferating cells.

Thus, according to a further aspect of the present invention
5 there is provided a method of determining grade or severity of neoplasia in a sample of tissue or tumor, the method comprising:

determining the number of blood vessels in the sample;
determining the number of blood vessels in the sample

10 that contain B-FN;

calculating the fraction of blood vessels in the sample that contain B-FN to provide an angiogenic index (AI) of the tissue or tumor, wherein the angiogenic index is indicative of the grade or severity of neoplasia in the tissue or tumor.

15

The number of blood vessels in a sample may be determined by means of staining and/or by means of a specific binding member for a blood vessel marker, e.g. Factor VIII. The number of blood vessels that contain B-FN may be determined using a
20 specific binding member for B-FN.

Generally, a specific binding member employed is an antibody molecule, and any suitable antibody molecule, label or labelling system may be used as disclosed herein in relation
25 to other aspects and embodiments of the present invention.

The AI may be correlated with grade or severity of neoplasia by means of comparison with known standards or controls, for instance the AI of tissues or tumors of known degree of
30 neoplasia.

The fraction of blood vessels containing B-FN is conveniently expressed as a percentage of the total number of blood vessels.

- 5 In Astrocytoma classified as high grade by conventional methods (Kleihues et al) more than 60% of the vessels contain B-FN whereas Astrocytoma classified as low grade contains no more than 10% B-FN positive vessels. Normal tissues contain no B-FN positive vessels, with the exception of the
- 10 Endometrium and the Ovary where angiogenesis is physiological.

Thus malignancy of a tumor may be established based on the AI as determined using the present invention.

- 15 All aspects and embodiments of the present invention may be used in diagnostic and prognostic situations. For prognosis, severity of disease may be determined and may be monitored with time, e.g. in the course of treatment or following completion of treatment to monitor for improvement or relapse.
- 20 The present invention may be readily applied to small biopsy samples obtained in the clinic thereby providing valuable information without additional compromise to the patient or complications for the clinicians. In the light of determinations using the present invention, clinical decisions
- 25 on intervention can be taken by appropriately qualified persons.

- Further aspects of the present invention relate to provision of novel antibody heavy and light chain variable regions, and
- 30 specific binding members comprising such a heavy and/or light chain variable region.

According to one aspect of the invention there is provided an antibody VH domain that consists of or comprises the amino acid sequence of SEQ ID NO. 2. Another aspect provides an antibody VL domain that consists of or comprises the amino acid sequence of SEQ ID NO. 4. A specific binding member comprising such a VH domain and/or VL domain provides a further aspect of the invention.

A further aspect provides an antibody antigen-binding site formed by association of the VH and VL domains of the invention. Antibody molecules providing such an antibody antigen-binding site are further provided, such as whole antibody, scFv and so on, as already discussed.

Further aspects provide nucleic acid encoding such a VH or VL domain, or antibody molecule such as scFv comprising these, and preferred embodiments consist of or comprise nucleic acid with the nucleotide sequence of SEQ ID NO. 1 (VH-encoding) or SEQ ID NO. 3 (VL-encoding).

The nucleic acid may be used in a method of production of the encoded polypeptide, in a suitable expression system such as an *in vitro* expression system, bacterial or eukaryotic host cell, in accordance with standard techniques.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NSO mouse melanoma cells and many others. A common, preferred bacterial host is *E. coli*.

The expression of antibodies and antibody fragments in prokaryotic cells such as *E. coli* is well established in the art. For a review, see for example Plückthun, A.

5 Bio/Technology 9: 545-551 (1991). Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of a specific binding member, see for recent reviews, for example Ref, M.E. (1993) Curr. Opinion Biotech. 4: 573-576; Trill J.J. et al. (1995) Curr. Opinion
10 Biotech 6: 553-560.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences,
15 enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press.
20 Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*,
25 Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

Thus, a further aspect of the present invention provides a
30 host cell containing nucleic acid as disclosed herein. A still further aspect provides a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells,

suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For
5 bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

The introduction may be followed by causing or allowing
10 expression from the nucleic acid, e.g. by culturing host cells under conditions for expression of the gene.

In one embodiment, the nucleic acid of the invention is integrated into the genome (e.g. chromosome) of the host cell.
15 Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

The present invention also provides a method which comprises
20 using a construct comprising nucleic acid as stated above in an expression system in order to express a specific binding member or polypeptide as above.

A method of production may comprise a step of isolation and/or
25 purification of the product.

A method of production may comprise formulating the product into a composition including at least one additional component, such as a pharmaceutically acceptable excipient.
30

Thus, the invention provides specific binding members comprising a VH and/or VL domain according to the present invention. A VH domain of the invention may be paired with

another VL, and a VL of the invention may be paired with another VH. Both a VH and a VL of the invention may be paired with one another.

5 Variants of the VH and VL domains of which the sequences are set out herein and which can be employed in specific binding members can be obtained by means of methods of sequence alteration or mutation and screening. Such methods are also provided by the present invention.

10

Variable domain amino acid sequence variants of any of the VH and VL domains whose sequences are specifically disclosed herein may be employed in accordance with the present invention, as discussed. Particular variants may include one
15 or more amino acid sequence alterations (addition, deletion, substitution and/or insertion of an amino acid residue), maybe less than about 20 alterations, less than about 15 alterations, less than about 10 alterations or less than about 5 alterations, 4, 3, 2 or 1. Alterations may be made in one
20 or more framework regions and/or one or more CDR's.

A specific binding member according to the invention may be one which competes for binding to antigen with any specific binding member which both binds the antigen and comprises a
25 specific binding member, VH and/or VL domain disclosed herein, or VH CDR3 disclosed herein, or variant of any of these.

Competition between binding members may be assayed easily *in vitro*, for example using ELISA and/or by tagging a specific reporter molecule to one binding member which can be detected
30 in the presence of other untagged binding member(s), to enable identification of specific binding members which bind the same epitope or an overlapping epitope.

In addition to antibody sequences, the specific binding member may comprise other amino acids, e.g. forming a peptide or polypeptide, such as a folded domain, or to impart to the molecule another functional characteristic in addition to ability to bind antigen. Specific binding members of the invention may carry a detectable label, or may be conjugated to a toxin or enzyme (e.g. via a peptidyl bond or linker).

Specific binding members according to the invention may be used in a method of treatment or diagnosis of the human or animal body, such as a method of treatment (which may include prophylactic treatment) of a disease or disorder in a human patient which comprises administering to said patient an effective amount of a specific binding member of the invention. They may be used in the manufacture of medicaments.

Additionally, they may be used in any of the various aspect of the present invention disclosed herein.

A VH or VL domain or specific binding member, or encoding nucleic acid, according to the present invention is generally provided in isolated form. This refers to the state in which specific binding members of the invention, or nucleic acid encoding such binding members, will be in accordance with the present invention. Members and nucleic acid will be free or substantially free of any material with which they are naturally associated such as other polypeptides or nucleic acids with which they are found in their natural environment, or the environment in which they are prepared (e.g. cell culture) when such preparation is by recombinant DNA technology practised *in vitro* or *in vivo*. Members and nucleic acid may be formulated with diluents or adjuvants and still for practical purposes be isolated - for example the members

will normally be mixed with gelatin or other carriers if used to coat microtitre plates for use in immunoassays, or will be mixed with pharmaceutically acceptable carriers or diluents when used in diagnosis or therapy. Specific binding members
5 may be glycosylated, either naturally or by systems of heterologous eukaryotic cells (e.g. CHO or NS0 (ECACC 85110503) cells, or they may be (for example if produced by expression in a prokaryotic cell) unglycosylated.

10 Further aspects and embodiments of the present invention will be apparent to those skilled in the art, given the present disclosure including the following experimental exemplification.

15 All documents referred to anywhere in this document are incorporated by reference.

EXAMPLE 1

*Quantitative detection of soluble FN, including A-FN and B-FN,
20 in tumor cell supernatant and plasma after immobilization onto gelatin.*

The inventors have utilized gelatin immobilization followed by immunodetection for the quantitative analysis of FN isoforms.
25 In this assay they have determined the sensitivity and specificity of A-FN and B-FN detection using calibrated, purified FN standards and corresponding recombinant proteins. The isoform containing the Extra Domain A was detected with the mouse monoclonal antibody IST-9 (Carnemolla, et al 1987)
30 and the isoform containing Extra Domain B was detected with the human recombinant anti-ED-B antibody (hrec anti-ED-B) (Figure 1). Total FN was detected with the anti-FN rabbit polyclonal antibody from Dako (U.S.A).

The quantitative detection of FN in plasma has been achieved using several different methods for divergent pathologies. The quantities of both total FN and A-FN have been investigated in relation to the presence of malignant disease (Boccardo et al. 1986; De Jager et al. 1996; de Jager et al. 1992; Haglund et al. 1997; Siri et al. 1983; Siri et al. 1984; Ylatupa et al. 1995a).

- 10 The inventors analyzed the plasma of mice and humans with the aim of quantitatively detecting total FN and the isoforms A-FN and B-FN in plasma. Furthermore, they analyzed the levels of B-FN expressed by cultured tumor cells which are routinely used in mouse tumor models. During this analysis they
- 15 determined the accuracy of A-FN and B-FN detection in plasma by blocking the specific signal with recombinant proteins and by supplementing plasma with known amounts of FN. They found that we were able to accurately, and quantitatively detect A-FN, B-FN and total FN in plasma and culture supernatant.
- 20 However A-FN cannot be quantified in mouse plasma because the anti-EDA antibody is a mouse monoclonal, and thus lacks immuno-reactivity against mouse A-FN.

MATERIALS AND METHODS

25

Cell lines sample preparation

The tumor cell line C51 (murine adenocarcinoma), and SK-MEL28 (human melanoma) were maintained in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS).

- 30 The cultures were plated at subconfluency (20,000 cells/cm²) and maintained for 10 days at which time they reached confluency. The culture supernatant was collected every 2 days and was supplemented with 0.1% NaN₃, 40 units Aprotinin/ml, 1mM

AEBSF (N-(2-aminoethyl)-benzenesulfonyl fluoride), centrifuged at 13000g to remove insoluble debris and subsequently frozen at -20°C.

5 Preparation of standards

FN was purified from fresh human plasma and WI38va13 cell culture medium using gelatin-sepharose affinity chromatography as previously described (Zardi et al. 1980). The absence of contaminants in purified FN was verified with FPLC and SDS-PAGE. The quantity of FN in purified FN aliquots was established by determining the OD₂₈₀ spectrophotometrically, and by total protein quantitation using the Protein Assay ESL from Boehringer Mannheim. Both plasma FN and WI38va13 FN standards were prepared by diluting purified FN to 2.5 µg/ml in PBST-BSA (Phosphate Buffered Saline; 0.05% Tween-20, 1% Bovine Serum Albumin). This solution was chosen since it is reported to prevent precipitation of FN (Ylatupa et al. 1995b). The solution was divided into 1 ml aliquots and maintained at -20°C. Before use each aliquot was melted at 37°C and centrifuged for 10 minutes at 13000g. The standard was diluted into PBST-BSA using serial two-fold dilutions thereby creating a range from 0.0003-1.25 µg/ml. Each aliquot was discarded after a single use. Purified plasma FN functions as a standard for the detection of total FN, human FN, and FN lacking ED-B and ED-A. In contrast, purified FN from WI38va13 functions as a standard for the detection of ED-A and ED-B containing FN.

FN immunoassay

96-well immuno-plates (Maxisorp, Nunc) were coated with 150 µl of 0.5% gelatin (Porcine skin, ~300 bloom, Sigma) in Phosphate Buffered Saline (PBS) for 4 hrs at 37°C. Non-specific sites were blocked with 300 µl blocking solution (3% Bovine Serum Albumin (BSA) in PBS, 2 hrs at 37°C). FN

containing solutions were diluted in PBST-BSA and 150 µl of appropriate dilutions was plated in duplicate wells and incubated overnight at room temperature. Subsequent detection of FN and specific FN isoforms was achieved by applying the appropriate antibody diluted in PBST-BSA (150 µl for 2 hrs at 37°C). For the detection of total FN, A-FN and B-FN we utilized polyclonal anti-FN (Dako, U.S.A.), monoclonal IST-9, and human recombinant anti-ED-B (hrec anti-ED-B) antibodies respectively (Figure 1). After the binding of the primary antibody, 150 µl of species specific biotinylated secondary antibody was applied for one hr followed by 150 µl of avidin/HRP complex (1 hr). Between each change of solution, the wells were washed 3 times with PBST. Specific binding was detected with 150 µl of the Peroxidase substrate TMP prepared as per instructions (Pierce, U.S.A). The reaction was stopped with the addition of 100 µl of 2 M Sulferic acid. The signal intensity was determined with the THERMOmax Microplate Reader (molecular devices, U.S.A.) at 450 nm and subsequent quantitative analysis was performed using SOFTmax PRO (molecular devices, U.S.A.) analysis software. Quantities of FN presented are the average of two adjacent dilutions analyzed in duplicate wells.

Plasma preparation

Human blood was collected from healthy donors through venal puncture into vacuum tubes containing EDTA to prevent clotting. Mouse blood was collected by cardiac puncture after chloroform anesthesia and transferred immediately to a tube containing EDTA to prevent clotting. Plasma was prepared by two consecutive centrifugations at 2000g and 13000g respectively. The plasma was supplemented with 0.1% NaN₃ and 20 u/ml aprotinin and subsequently frozen at -80°C.

RESULTS

The maximum sensitivity of this assay was found to be 2.5 ng/ml, 1 ng/ml, and 0.3 ng/ml for the detection of A-FN, B-FN and total FN respectively (Figure 2). Specific binding of the isoform specific antibodies IST-9 and hrec anti-ED-B were blocked completely by the addition of the corresponding recombinant protein ED-A or ED-B.

Quantitative analysis of culture supernatants from C51 and SK-MEL 28 confirmed that these cells, in confluent cultures, produce 2.5 and 12.5 µg/ml in two days. Furthermore, both C51 and SK-MEL 28 produced B-FN at 2.5 and 5.6 µg/ml each (Table I). The media of Wi38va13 cells which is used routinely as a source of B-FN was used as a positive control. In 2 days of confluent culture, this cell line produces 3µg/ml of FN, all of which contains the ED-B.

Our analysis of a population of control individuals demonstrates that human plasma (n=29) contains approximately 5.7 ± 2.5 µg/ml A-FN, 1.03 ± 0.25 µg/ml of B-FN and 317 ± 59.6 µg/ml total FN (Table I and Figure 3). It is clear that the proportion of plasma FN that contains the Extra Domain B is much smaller than the proportion that contains the Extra Domain A (0.34 ± 0.09% vs. 1.8 ± 0.74%). Furthermore the level of B-FN is more stable within the control population than A-FN as is reflected in an analysis of variation which provides a %CV of 43.9 for A-FN and 24.6 for B-FN (See Table IIA and Figure 3).

In contrast to human plasma, our analysis of mouse plasma demonstrates that B-FN is found to be approximately 0.061 ± 0.013 µg/ml for BALB/c mice and 0.12 ± 0.017 for Nude mice. Total FN, as compared to a human FN standard, is present at

levels of 163 ± 15 $\mu\text{g/ml}$ for BALB/c mice and 213 ± 24 $\mu\text{g/ml}$ for nude mice (Table I). Mouse A-FN could not be quantified in mouse plasma, using the antibody the inventors had in the laboratory, because of the species conflict for the mouse
5 monoclonal antibody IST-9. Clearly, on the basis of the results presented herein an antibody of a different species is expected to be useful in accordance with the invention for detection of mouse B-FN in mouse plasma.

10 The accuracy of A-FN and B-FN detection in plasma was confirmed by the recovery of known quantity of purified standard FN supplemented to four separate plasma samples. The recovery of this supplemented A-FN and B-FN was $100 \pm 4.6\%$ and $95.5 \pm 1.8\%$ respectively. The specificity of detection was
15 confirmed by blocking of specific binding with competitive recombinant ED-A and ED-B.

EXAMPLE 2

*Determination of elevated levels of B-FN in tumor bearing mice
20 and in cancer patients.*

B-FN was found to be more frequently and specifically elevated in cancer patients than either A-FN or total FN.

25 Total FN and A-FN have already been investigated for the use as a marker of malignant disease (Boccardo et al. 1986; Dreyfus et al. 1998; Haglund et al. 1997; Katayama et al. 1991; Ruelland et al. 1988; Siri et al. 1984; Ylatupa et al. 1995a). In comparison, the inventors undertook an
30 investigation of tumor bearing mice, and of a population of control individuals ($n=29$) and cancer patients ($n=64$) to compare the use of A-FN and B-FN as markers of malignant disease. Both isoforms were compared with the total quantity

of plasma FN to illustrate the relative proportion of each isoform.

Using the immunoassay described in Example 1 the inventors
5 monitored the level of B-FN and total FN in the plasma of mice
bearing the murine adenocarcinoma C51 or human melanoma SK-MEL
28 to determine circulating levels of these proteins. It was
found that B-FN was specifically elevated in mice bearing C51
tumors. This observation was confirmed in animals bearing SK-
10 MEL 28.

As already described in Example 1, it was found that B-FN is
present at much lower levels in control plasma than A-FN and
has a lower variability within the control population. An
15 analysis of the cancer patient population demonstrated that B-
FN was elevated more frequently and more specifically than A-
FN. Furthermore, it was found that the frequency of B-FN
elevation is higher for some tumors than for others (Table IIB
and Figure 5). These data provide indication that the analysis
20 of B-FN by gelatin immobilization ELISA can be utilized to
assist in the diagnosis of tumor patients.

MATERIALS AND METHODS

25 Cell lines and Mouse tumor models

The cell lines were maintained as described in Example 1. C51,
and SK-MEL-28 cells were trypsinized, resuspended in PBS,
counted, and injected subcutaneously into mice. At indicated
time points the animals were sedated with chloroform and blood
30 was collected by cardiac puncture.

Immunoassay and analysis of plasma

Plasma from mice bearing tumors was prepared as described in Example 1. The immunoassay was performed as described in Example 1.

5 Data processing and statistical analysis

Data obtained from the quantitative immunoassay were transferred from the Softmax Pro software to Microsoft Excel where the data were grouped and the mean, standard deviation and coefficient of Variance (CV%) were determined. The grouped
10 data were transferred to the Systat statistical program for non-parametric statistical analysis. As established in the literature, 97.5% of the control was used as cutoff for the distinction between elevated levels and control levels (Ylatupa et al. 1995a).

15

RESULTS

Plasma from mice bearing C51 tumors was found to contain elevated levels of B-FN (Figure 4A). In the same mice the level of total FN was also elevated. However the proportional
20 elevation of B-FN exceeded the total FN elevation indicating a specific increase in the %B-FN (Figure 4B). An analysis of plasma from mice bearing human tumor cells SK-MEL 28 confirmed our findings in the syngenic C51 mouse model.

25 As discussed in Example 1, the level of B-FN in control individuals was lower and more stable than A-FN. The 97.5% cutoff limit for A-FN, B-FN and total FN was determined to be 1.53 µg/ml, 11.1 µg/ml, and 427 µg/ml respectively (Table IIA). The cancer patient population was subdivided into groups
30 based on tumor type (Table IIB). It was found that in the entire tumor population (n=64) 46% of the individuals had elevated levels of B-FN whereas A-FN and total FN were elevated in only 17% and 9% of the population. Furthermore, it

was determined that among the different tumor types there were striking differences in the frequency of elevation. B-FN was elevated in 16.6% intra cranial tumor patients (n=24, p=0.28), 71% of melanoma patients (n=17, p=0.004), and 100% of breast carcinoma patients (n=7, p=0.00005) (Table IIB). A-FN and total FN demonstrated a similar pattern of tumor type specific elevation but with a much lower frequency of elevation.

To analyze the specificity of A-FN and B-FN elevation the inventors determined the percentage of each isoform with respect to total FN (Table IV). Specific elevation is clearly shown for B-FN (% ED-B = $0.44 \pm 0.2\%$, p=0.04) but not A-FN (% ED-A = $1.99 \pm 1.3\%$, p=0.9) (Figure 5).

The experiments demonstrate that by gelatin immobilization ELISA FN and its tumor associated isoforms, in particular B-FN, can be quantitatively detected in plasma. Using this immunoassay it has been demonstrated that B-FN is a more frequent and specific marker for malignant disease than A-FN. Furthermore, the elevation of B-FN appears to correlate to the type of tumor.

EXAMPLE 3

Quantitative detection of FN in cerebral-spinal and cystic fluid using Gelatin immobilization ELISA.

Although the analysis of tumor markers is most common for plasma samples, the same analysis can be useful for ascites fluid, cystic fluid, cerebral-spinal fluid, synovial fluid, seminal fluid and lymphnode aspirates (Carnemolla et al. 1984; Gomez-Lechon and Castell 1986; Leon et al. 1986; Rieder et al. 1991; Siri et al. 1984; Ylatupa et al. 1995a). To demonstrate that the described methodology of gelatin immobilization ELISA

can be applied in other bodily fluids, several cystic fluids and cerebral-spinal fluids were analysed.

FN, including the tumor associated isoforms A-FN and B-FN were
5 readily detected in these fluids.

MATERIALS AND METHODS

Immunoassay and analysis of fluid

10 Cerebral-spinal and cystic fluid were obtained from cancer patients during scheduled surgeries and prepared as described for plasma in Example 1. The immunoassay was performed as described in Examples 1 and 2.

15 *RESULTS*

Table III illustrates the quantitative results from cerebral-spinal and cystic fluid samples analyzed by gelatin immobilization ELISA. The sensitivity of the assay combined
20 with the use of serial dilutions allows for accurate quantitation of FN in the cerebral-spinal and cystic fluids which contain much greater variation than plasma. Note that those samples which contain very high levels of B-FN (>10µg/ml) were obtained from metastatic malignancies while
25 samples from primary intra-cranial tumors contained much lower quantities of B-FN. Such data can provide useful diagnostic information in the clinic and can assist in enhancing the evaluation of individual patients.

30 *EXAMPLE 4*

Quantitatively detection of insoluble B-FN in tumor tissues from mouse tumor models

The inventors devised a procedure to achieve quantitative detection of B-FN, employing an I^{125} radiolabeled human recombinant antibody ("L19") directed against the Extra Domain B with quantification of its incorporation into tissue homogenate. In the procedure according to the invention, the antibody bound to the insoluble B-FN is separated from unbound antibody, e.g. by a vacuum filtration system, and the remaining label (e.g. radio-activity) is determined (e.g. using a standard gamma counter). A B-FN standard was generated by chemical cross-linking of Wi38va13 FN to Sepharose and used in parallel to the unknown tissue samples. The raw data were imported into analytical software which plots the calibration curve and interpolates the radio-activity immobilized in the tissue with the standard curve. The analysis results are presented in μg of B-FN/gram fresh tissue.

MATERIALS AND METHODS

Radiolabeling of antibodies with I^{125}

Radiolabeling of antibodies was performed using IODO-GEN® pre-coated tubes (Pierce, Rockford, USA) using the protocol provided by Pierce. Antibodies were radio-labeled with I^{125} at 2 mCi/mg of protein for a total of 7 minutes of labeling. The labeled material was separated from the free I^{125} by passing the reaction mixture over a PD-10 column. The specific activity of the labeled protein was determined based on the activity in peak fractions after considering a 20% loss of protein (generally 0.3-0.5 $\mu\text{Ci}/\mu\text{g}$). The retention of immuno-reactivity was determined as follows: (1) 10 μl of labeled material was added to a small column of ED-B sepharose prepared in a 1000 μl pipette tip; (2) the unbound material was removed with PBS washes and the bound material recovered with a 1.5 ml elution of 0.1M TEA (Triethanolamine) at pH 11.

The immuno-reactivity is expressed as the % of labeled material that bound to the column and was eluted using TEA (generally between 85-95%).

5 Preparation of Wi38va13 FN standard

A reproducible standard of insoluble B-FN was obtained by crosslinking cellular FN purified from Wi38va13 culture medium to 4B CNBr-Sepharose. Wi38va13 FN was purified by Gelatin affinity chromatography (as described by Zardi et al. 1980, and in Section I, Example I). The proportion of FN containing ED-B was found to be >95% as determined by ELISA. Prior to crosslinking, FN was thawed at 37°C and centrifuged at 13000g to remove precipitates. The FN was crosslinked to 4B CNBr-Sepharose as per instructions from the vendor and stored at 15 4°C in PBS, 3% BSA, 40 U Aprotinin/ml, 1mM AEBST, 1mM EDTA and 0.1% NaN₃.

Tissue preparation

Mouse tissue was obtained by surgical removal of tumor or 20 normal tissue from sacrificed mice. The murine teratocarcinoma tumors (F9) were prepared by subcutaneous injection of tumor cells. In contrast, the murine adenocarcinoma (C51) and human neuroblastoma cells were injected intravenously to allow for establishment of 25 metastasis.

Fresh and frozen tissues were prepared for quantitative analysis by homogenization with a Dounce homogenizer. Frozen tissues were first thawed in PBS and rinsed 2X prior to 30 homogenization. Tissues were weighed and subsequently minced using a scalpel. This material was transferred to a Dounce homogenizer containing 1 ml of buffer (PBS, 3% BSA, 40 U Aprotinin/ml, 1mM AEBST, 1mM EDTA) for every 200 mg tissue and

homogenized using a Teflon plunger. The tissue was homogenized until a homogenous mixture was obtained. The homogenate was diluted in 8 serial 2 fold dilutions starting at 100 mg/ml.

5 Quantitative radio-immunological detection of insoluble B-FN

200 µl of each homogenate dilution was transferred to an Eppendorf tube. 300,000 CPM of L19-I¹²⁵ was added to the dilutions containing 100, 25, and 6.25 mg of tissue/ml, while 300,000 CPM of IST-4-I¹²⁵ was added to the dilutions containing
10 50, 12.5, 3.125, and 0.78 mg of tissue/ml. Each dilution was performed in duplicate. The resulting mixtures were incubated on a vertical rotator at RT for 2.5 hr (binding saturated at 2hr). The tubes were subsequently placed in a rack and two
15 holes introduced into the cap using an 18 gauge needle. Into one hole was introduced an 18 gauge needle connected to a 40 ml reservoir of PBS via a piece of tubing. Into the other hole was introduced a pipette tip (1000µl) connected to a filter gasket via a piece of tubing. The filter gasket contained a glass filter and was attached directly to a vacuum flask. When
20 the vacuum was initiated, the reservoir of PBS was drawn through the Eppendorf containing the homogenate/antibody mixture thereby transferring it onto the glass filter and pulling the soluble material including unbound antibody into the vacuum flask. A secondary wash of PBS (10ml) was drawn
25 through the filter to remove any residual unbound antibody. The glass filter was subsequently removed and transferred to gamma counter tube.

To demonstrate specificity of binding, radio-labeled L19 anti-
30 ED-B antibody was pre-incubated with a 400 fold molar excess of recombinant ED-B protein to block specific binding to insoluble B-FN in the tissue homogenate (Figure 6B).

To generate a standard curve (ranging from 10-0.01 μ g) radio-labeled antibody was incubated with the FN-Sepharose described above and transferred onto glass filters in the same manner as described for the tissue homogenate (Figure 6A).

5 Specific binding was determined by the detection of gamma radiation immobilized onto the glass filters using a gamma counter. The data obtained from this reading were entered into a excel spread sheet and subsequently transferred to the
10 Softmax Pro analysis software. In this analytical program we completed regression analysis of the standard and interpolated the signal from the tissue homogenates to determine the relative quantity of insoluble B-FN (Figure 6A).

15 RESULTS

The standard curve generated by the detection of B-FN crosslinked to sepharose demonstrates that the method can detect as little as 100 ng/sample while generating a quantitative detection range between 0.1 and 10 μ g (Figure 6).

20 The analysis of normal mouse tissues demonstrated that no insoluble B-FN could be detected in liver or lung. In contrast B-FN could be detected in various mouse tumors at levels ranging from 100 to 500 μ g/gram of tissue (Table IV).

25 Specificity of detection was confirmed by blocking with recombinant ED-B (Figure 6B).

EXAMPLE 5

30 Quantitative detection of insoluble B-FN in human tissues; a comparison of B-FN in human tumors and mouse tumor models and a correlation between the quantity of insoluble B-FN and the grade of tumor.

Using the radio-immunoassay described in Example 4 it was determined that while B-FN is undetectable in normal human tissues, it is readily detectable in tumor tissues (Table IV). Furthermore, a correlation was found between the quantity of B-FN and the grade of astrocytoma, providing indication of a relationship between the grade of malignancy and the quantity of B-FN present.

MATERIALS AND METHODS

Tissue preparation and radio-immunological detection

The tissue was prepared and analyzed as described in Example 4. All of the human tissue was obtained as material embedded in mounting media for cryosectioning. To provide an internal comparison, the anti-FN antibody IST-4 was radiolabeled with I^{125} and detection of B-FN was compare with the detection of total FN.

RESULTS

Using the radio-immunoassay described above, insoluble B-FN was undetectable in normal lung tissue (n=6) and normal brain tissue (n=3). However the same tissue was found to contain detectable levels of total FN with a range of 60-130 $\mu\text{g}/\text{gram}$ for lung and 4.6-6.3 $\mu\text{g}/\text{gram}$ for brain tissue (Figure 7A and Table IV).

Normal lung tissue contains no detectable B-FN but substantial levels of total FN (Figure 7A). In contrast tumor lung tissue contains B-FN and elevated levels of total FN. Normal brain tissue was found to contain no detectable levels of B-FN and very low levels of total FN (Figure 7B). In contrast the tissue from high grade Astrocytoma contained substantial amounts of B-FN and greatly elevated levels of total FN. A

comparison between high and low grade Astrocytoma illustrates the correlation between the tumor grade and the presence of B-FN and total FN (Figure 7B).

5 An analysis of lung tumors (n=11, Figure 4A) showed that B-FN was present at 143 ± 89 $\mu\text{g}/\text{gram}$. The same tissue contained 341 ± 78 $\mu\text{g}/\text{gram}$ of total FN (Figure 7A and Table IV). Both the elevation of total FN and the detection of B-FN demonstrate statistical significance (Mann Whitney $p=0.005$ and 0.0006
10 respectively).

Two groups of astrocytoma samples were analyzed to determine if a correlation existed between the tumor grade and the quantity of B-FN present in the tumor tissue (Figure 7B). Low
15 grade astrocytoma samples were found to contain 23 ± 28 μg B-FN/gram and 19 ± 10 μg total FN/gram. In contrast high grade astrocytoma tissue contained 219 ± 142 μg B-FN/gram and 239 ± 181 μg total FN/gram (Table IV and Figure 7B). Non-parametric statistical analysis verifies that the two tumor populations
20 differ significantly both in total FN ($p=0.0004$) and B-FN ($p=0.0004$).

A comparison of mouse tumor models carcinoma with human tumors illustrates that the similar quantities of B-FN found in both
25 tumor tissues. Human lung tumors and high grade astrocytomas contain quantities of B-FN that range from 50-400 $\mu\text{g}/\text{gram}$ tissue, while the tumor tissue from 3 different mouse tumor models contains approximately 100-500 $\mu\text{g}/\text{gram}$ respectively (Table IV). The similarity between the mouse tumor models and
30 the human tissue allows for assessment of the effectiveness of B-FN based targeting and therapy in humans.

The methodology described provides a novel method for the quantitative detection a tumor associated protein which is an insoluble component of the tumor microenvironment. This methodology provided invaluable information for translation of knowledge obtained from mouse tumor models to humans in the clinic. Although the technique is established for the detection of B-FN, it is readily applicable to other insoluble components of the tumor microenvironment. Tumor tissues contain a number of identified components which are insoluble and may be quantified in a similar manner. Among such antigens are A-FN, the tumor specific splice variants of Tenascin, and Laminin, which are insoluble components of the tumor tissue.

EXAMPLE 6

Analysis of the vascular density, the angiogenic index, and cellular proliferation in cryostat sections from high and low grade Astrocytoma.

The inventors evaluated the percentage of B-FN reactive vessels in cryosections of Astrocytic tumors by double immunostaining using a monoclonal antibody (MAb) specific for Factor VIII, an endothelial cell marker, and a human recombinant antibody specific for the FN domain ED-B. The results (Table V and VI, and Figure 8) show that the percentage of B-FN positive vessels correlated with the grade of the tumor. In all anaplastic Astrocytomas (grade III and Glioblastomas), more than 60% of the blood vessels showed intense and extensive staining with the recombinant anti-ED-B antibody. Conversely, in low grade (I and II) Astrocytomas, it was found that no more than 10% of all vessels showed positivity when reacted with the recombinant anti-ED-B. Adjacent portions of brain ranging from normal to areas of

reactive gliosis consistently showed only B-FN negative vessels.

MATERIALS AND METHODS

5

Tissues

Normal and neoplastic tissues were obtained from samples taken during the course of therapeutic surgical procedures. The samples investigated included 28 low-grade Astrocytomas and 28
10 anaplastic Astrocytoma and Glioblastomas (see Tables V and VI). Each sample was divided into 2 parts: one was processed for conventional histopathological diagnosis and the other was immediately frozen in liquid nitrogen. Cryostat sections 6 um thick were stained with hematoxylin-eosin, and additional
15 frozen sections were used for immunohistochemical staining after fixation in absolute cold acetone for 10 min. To prevent the heterogeneous distribution of FN within the tissues from giving false-negative results, at least 3 non-consecutive sections of the biopsy were analyzed. Astrocytic tumors were
20 graded and classified according to Kleihues et al. (1993).

Immunohistochemical procedures

For immunohistochemical studies, 6um thick cryostat sections were air-dried and fixed in cold acetone for 10 min.
25 Immunostaining was performed using a streptavidin-biotin-alkaline-phosphatase-complex staining kit (Bio-Spa Division, Milan, Italy) and naphtol-AS-MX-phosphate and Fast-Red TR (Sigma, St. Louis, MO) to visualize binding sites. Gill's hematoxylin was used as a counterstain, followed by mounting
30 in glycergel (Dako, Carpinteria, CA).

Double-staining experiments were performed according to Sternberger and Shirley (1979). The first reaction sequence

consisted in the application of the primary MAb, incubation with biotinylated goat anti-mouse IgG (Bio-Spa) and then with avidin-biotinylated-peroxidase complex (Bio-Spa).

Immunoenzymatic staining was then carried out using 3,3-
5 diaminobenzidine tetrahydrochloride (DAB)-H₂O₂ (Sigma), which yielded a brown reaction product masking antigens and immunoreagents of this first sequence and thus preventing cross-binding of the antibodies in the second sequence. Next, the sections were incubated with the second primary MAb, then
10 incubated with the secondary antibody and streptavidin-biotinylated-alkaline-phosphatase complex (Bio-Spa). The red reaction product was obtained using a mixture of 2mg naphthol-AS-MX phosphate (Sigma) dissolved in 200µl of n,n-dimethylformamide (Sigma) and diluted in 9,8 ml of 0.1M Tris-HCl buffer, pH 8.2, and 1Mm levamisole (Sigma). Immediately
15 before use, 10mg of Fast-Red TR salt (Sigma) were added. Gill's hematoxylin was used as a counterstain, and the sections were mounted in glycerol (Dako). The number of KI-67 positive cells, the number of Factor VIII positive vessels and the
20 number of B-FN positive vessels were determined by counting five different high magnification fields (HMF) of each specimen at 40X microscopic magnification.

Monoclonal antibodies (Mabs) and human recombinant antibodies

25 The anti-human Factor VIII MAb (Dako-Factor VIII) and the anti-KI-67 MAb specific for proliferating cells were purchased from Dako (Carpenteria, CA). The Mab 9E10 to the Myc tag peptide was purchased from ATCC, Rockville, MD. The human recombinant antibody, specific for the ED-B domain, has been
30 previously described (Carnemolla et al. 1996).

RESULTS

The vascular density as well as the angiogenic index (by means of the percentage of B-FN positive vessels) and number of proliferating cells was evaluated in all the specimens. The results are shown in Tables V and VI, and Figures 8A-C. These results show that while within the 2 groups of high and low grade Astrocytoma there are overlapping results for what concerns the vascular density and number of proliferating cells (Figure 8A and C), there is not any overlap in the percentage of FN-B positive vessels (Figure 8B).

The observations provide indication that the determination of the angiogenic index, through immunohistochemical detection of the percentage of B-FN positive vessels, is useful for supplementing and refining the conventional grading system of Astrocytomas. It may also help in their histological grading, and in the differential diagnosis between some astrocytomas and reactive gliosis.

Furthermore, since the several studies have already demonstrated that a tumor angiogenesis is a prognostic indicator for many tumors, the AI (as the percentage of B-FN positive vessels) may be useful as an adjunct tool in the diagnosis and prognosis of cancer patients. In addition, the easy applicability of this adjunct tool to cryosections may be used in evaluating small biopsy samples.

EXAMPLE 7

Generation of antibody molecules specific for ED-B

30

The method of Skerra et al. was used to isolate antibody ME4C, of which the VH and VL amino acid sequences and encoding nucleotide sequences are disclosed herein. The synthetic

antibody library ETH-2 (<http://www.pharma.ethz.ch/bmm/>) was employed. Bacterial colonies expressing antibody fragments were grown on a master porous filter, in contact with a second filter coated with the ED-B domain of fibronectin. Antibody fragments diffusing onto the second filter which specifically recognise the antigen were detected with a monoclonal antibody directed against a FLAG-tag sequence, appended at the carboxy terminal extremity of the antibody fragment.

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Table I. Detection of B-FN and total FN in human and mouse plasma, and tumor cell culture medium using gelatin immobilization ELISA

	Plasma			Culture supernatant	
	Human*	BALB/c mice*	Nude mice*	C51 [^]	SK-MEL 28 [^]
A-FN	5.7 . 2.5	N.D.	N.D.	N.D.	N.D.
B-FN	1.03 . 0.25	0.061 . 0.013	0.12 . 0.017	2.4	5.4
Total FN	317 . 59.6	163 . 15	213 . 24	3	12

* = µg FN/ml plasma. Human n= 29, BALB/c n=6, Nude n=3.

[^] = µg FN/ml in culture supernatant after 2 days.

N.D.= Not Determined

Table II. Quantitative analysis of A-FN, B-FN and total FN in plasma from control individuals and cancer patients.

A)

Control (n=29)	µg/ml A FN	µg/ml B FN	µg/ml total FN	% A-FN	% B-FN
average . Stdev (CV%)	5.7 . 2.5 (43.9)	1.03 . 0.25 (24.6)	317 . 59.6 (18.8)	1.8 . 0.74 (41.3)	0.34 . 0.09 (28)
range (97.5 percentile)	2.3-11.9 (11.1)	0.5-1.7 (1.53)	207-456 (427)	0.79-3.5 (3.21)	0.14-0.59 (0.56)

B)

Tumor type (n)	A-FN		B-FN		total FN		% A-FN		% B-FN	
	Mean \pm stdev	Elevated (#/%)	Mean \pm stdev	Elevated (#/%)	Mean \pm stdev	Elevated (#/%)	Mean \pm stdev	Elevated (#/%)	Mean \pm stdev	Elevated (#/%)
Intra cranial (24)	5.2 \pm 3.8	3/12.5	1.1 \pm 0.65	4/16.6	308 \pm 74	0/0	1.66 \pm 1	3/12.5	0.32 \pm 0.18	3/12.5
Melanoma (17)	7.5 \pm 3.1	3/18	1.6 \pm 0.6	12/71	367 \pm 78	2/12	2 \pm 0.72	1/6	0.45 \pm 0.16	6/35
Breast Cancer (7)	8.7 \pm 6.7	3/43	2.3 \pm 1	7/100	430 \pm 156	4/57	1.9 \pm 1.02	1/14	0.58 \pm 0.2	4/57
Cancers of the Reproductive System(5)	5.3 \pm 1	0/0	1.3 \pm 0.47	2/40	318 \pm 44.5	0/0	1.9 \pm 0.26	0/0	0.43 \pm 0.18	2/40
Renal and Bladder Cancer (4)	5.7 \pm 1.3	0/0	2.2 \pm 1.1	2/50	320 \pm 36	0/0	1.8 \pm 0.27	0/0	0.7 \pm 0.42	2/50
Lung Cancer (2)	9.3 \pm 5.7	1/50	2.2 \pm 1	1/50	356 \pm 0	0/0	2.61 \pm 1.6	1/50	0.62 \pm 0.28	1/50
Other(5)	15.3 \pm 10.6	2/40	1.6 \pm 0.6	2/40	362 \pm 46	0/0	4.1 \pm 2.5	2/40	0.44 \pm 0.13	1/20
Total (64)	7.06 \pm 5.5	12/18.8	1.5 \pm 0.8	30/46	341 \pm 89	6/9	1.99 \pm 1.3	8/12.5	0.44 \pm 0.2	19/29.6

CV% = coefficient of variance calculated as $\text{stdev}/\text{mean} \times 100$, stdev = Standard Deviation, # refers to the number of individuals elevated, n = total number of individuals, % refers to the percentage of individuals elevated.

Table III. Detection of FN in cerebral spinal and cystic fluid using gelatin immobilization ELISA.
Cerebral Spinal Fluid

Diagnosis	µg/ml A-FN	µg/ml B-FN	µg/ml FN	% A-FN	% B-FN
Glioblastoma	0.65	0.321	1.25	51.8	25.56
Glioblastoma	5.34	3.393	23.8	22.4	14.26
Meningioma	0.462	0.163	12.9	3.6	1.262
Unknown metastasis	0.92	0.66	2.28	40.1	28.7
Lung cancer metastasis	12.55	20.69	121.52	10.3	17.0
Astrocytoma I. Pilocytic	0.242	0.115	1.667	14.5	6.9
Glioblastoma	0.31	0.369	5.3	5.89	6.954

5 Cystic Fluid

Diagnosis	µg/ml A-FN	µg/ml B-FN	µg/ml FN	% A-FN	% B-FN
Lung cancer metastasis	58.1	21.5	212	27.3	10.1
Colon Carcinoma metastasis	13.37	23.59	142.96	9.4	16.5
Astrocytoma I. Pilocytic	2.31	0.82	31.73	7.3	2.6

Table IV. Quantitative detection of B-FN in normal and tumor tissue from mice and humans.

Mouse	B-FN ($\mu\text{g/g}$. tissue)	Human	B-FN ($\mu\text{g/g}$. tissue)
Normal lung	N.D.	Normal lung	N.D.
Normal liver	N.D.	Lung tumors	143 \pm 89
Normal kidney	N.D.	Normal brain	N.D.
C51 Lung Metastasis	500	Glioma. low grade	23 \pm 28
F9 teratocarcinoma	333	Glioma. high grade	219 \pm 142
Neuroblastoma	125		
Metastasis			

5 N.D.= Not Detectable

Table V. Low grade astrocytomas

Case	Diagnosis ¹	% of EDB positive vessels ²	Number of vessels per HMF ³	Number of Ki67 positive cells per HMF ⁴
1	astrocytoma I (fibrillar)	1.0	57.0	8.0
2	astrocytoma II	0.0	18.0	6.0
3	astrocytoma II	0.0	34.0	0.0
4	astrocytoma II	0.0	15.0	0.0
5	astrocytoma II	0.0	20.8	0.0
6	astrocytoma I	0.0	28.0	0.0
7	astrocytoma I/II	0.0	14.0	0.0
8	astrocytoma II	0.0	13.6	2.2
9	astrocytoma II	0.0	15.4	2.4
10	astrocytoma II	0.0	37.6	0.6
11	astrocytoma I	0.0	14.0	0.0
12	low grade ganglioglioma	0.0	3.8	0.4
13	low grade ganglioglioma	0.0	5.0	1.2
14	pilocytic astrocytoma	1.5	12.2	0.2
15	pilocytic astrocytoma	3.9	14.4	0.0
16	astrocytoma I/II	4.8	20.6	0
17	astrocytoma I/II	1.9	21.6	0.2
18	astrocytoma II	0.0	7.6	0.0
19	pilocytic astrocytoma	5.7	29.0	0.0

20	DNE*	4.0	4.4	2.6
21	oligodendrioglioma	2.0	24.6	0.4
22	DNE*	3.7	8.8	0.0
23	astrocytoma II	0.0	5.2	0.0
24	astrocytoma II	0.0	6.8	0.6
25	astrocytoma II	9.6	15.8	3.6
26	DNE*	8.0	24.0	1.0
27	astrocytoma II/ oligofibrillar glioma	0.0	7.6	0.0
28	DNE*	0.0	22.6	0.0
average \pm SD		1.6 \pm 2.7	17.9 \pm 11.8	1.1 \pm 2.0

¹ Diagnosis was established by examining formalin fixed, paraffin embedded and hematoxylin-eosin stained sections of 6 μ m

*DNE: dysembryoplastic neuroepithelial tumor

² Percentage of ED-B positive vessels was calculated by dividing the number of EDB positive vessels by the number of factor-VIII positive vessels counted on frozen sections of 6 μ m, double stained for EDB fibronectin and factor-VIII related antigen. For each case one slide was examined. Five high magnification fields (HMF) of 25X were examined; numbers represent average of the five counts.

³ Number of vessels per field were counted on frozen sections of 6 μ m stained for factor-VIII related antigen. For each case one slide was examined. Five high magnification fields (HMF) of 25X were examined; numbers represent average of the five counts.

⁴ Number of Ki67 positive cells per field were counted on frozen sections of 6 μ m stained for Ki67. For each case one slide was examined. Five high magnification fields (HMF) of 40X were examined; numbers represent average of the five counts.

Table VI. High grade astrocytomas

Case	Diagnosis ¹	% of EDB positive vessels ²	Number of vessels per HMF ³	Number of Ki67 positive cells per HMF ⁴
1	glioblastoma	87	24.4	20
2	glioblastoma	74.4	95	6.2
3	glioblastoma	92.7	90	34
4	glioblastoma	85	21	1.4
5	astrocytoma III	97.2	72	18
6	astrocytoma III	99.2	123	28
7	glioblastoma	92.8	29	17.4
8	astrocytoma III	99.2	29	18.4
9	glioblastoma	96.8	14.4	6.8
10	astrocytoma III (gemistocytic) / glioblastoma	93.5	63.7	73.4
11	glioblastoma	76.7	27.2	117.4
12	glioblastoma	92.1	26.6	12.6
13	astrocytoma IV (gemistocytic)	100	42.8	25.6
14	astrocytoma III	84.7	73.2	64.2

15	Glioblastoma	96.8	12.2	47.6
16	astrocytoma III / glioblastoma	89.3	39.4	52.4
17	anaplastic astrocytoma	86.8	19.2	55
18	astrocytoma III	92.6	24	36.4
19	glioblastoma	70.5	34	1.2
20	glioblastoma	97	17.4	84.2
21	glioblastoma	98.8	60.4	40.2
22	glioblastoma	76.3	20	31.2
23	anaplastic astrocytoma	100	22.2	32.2
24	anaplastic astrocytoma	99	29.2	32
25	anaplastic astrocytoma	96.7	27.4	
26	glioblastoma	100	13.8	
27	glioblastoma	100	63.2	30.2
28	Glioblastoma	100	26	19
average \pm SD		92.0 \pm 8.7	40.7 \pm 28.4	34.2 \pm 26.8

¹ Diagnosis was established by examining formalin fixed, paraffin embedded and hematoxylin-eosin stained sections of 6 μ m

² Percentage of ED-B positive vessels was calculated by dividing the number of EDB positive vessels by the number of factor-VIII positive vessels counted on frozen sections of 6 μ m, double stained for EDB fibronectin and factor-VIII related antigen. For each case one slide was examined. Five high magnification fields (HMF) of 25X were examined; numbers represent average of the five counts.

3 Number of vessels per field were counted on frozen sections of 6 μ m stained for factor-VIII related antigen. For each case one slide was examined. Five high magnification fields (HMF) of 25X were examined; numbers represent average of the five counts.

4 Number of Ki67 positive cells per field were counted on frozen sections of 6 μ m stained for Ki67. For each case one slide was examined. Five high magnification fields (HMF) of 40X were examined; numbers represent average of the five counts.

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15

SEQ ID NO. 2

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SEQ ID NO. 4

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AEDEADYYCNSSA
10 PVSNRVVFEGGKTK

CLAIMS:

1. A method of determining the presence of fibronectin isoform B (B-FN) in a body fluid taken or collected from an individual, the method comprising:
 - passing the body fluid over a solid support to which is attached gelatin, able to bind fibronectin, whereby fibronectin, if present in the body fluid, is bound by the gelatin and retained on the solid support;
 - determining the presence or absence of B-FN retained on the solid support, by determining the ability of a specific binding member for the Extra Domain B (ED-B) of B-FN to be retained on the solid support.
2. A method according to claim 1 wherein the determination of the presence of B-FN is quantitative.
3. A method according to claim 1 or claim 2 which further comprises determination of the presence of a second isoform of fibronectin, by determining the ability of a specific binding member for said second isoform to be retained on the solid support.
4. A method according to claim 3 wherein determination of the presence of the second isoform of fibronectin is quantitative.
5. A method according to claim 3 or claim 4 wherein the second isoform of fibronectin is fibronectin isoform A (A-FN).
6. A method according to any one of the preceding claims wherein the specific binding member for ED-B is an antibody molecule.

7. A method according to any one of claims 3 to 6 wherein the specific binding member for the second fibronectin isoform is an antibody molecule.

5

8. A method according to any one of the preceding claims wherein a specific binding member is labelled with a radioisotope, an enzyme and/or a fluorescent moiety.

10 9. A kit for use in a method according to any one of the preceding claims which comprises

i) a solid support to which is bound gelatin able to bind fibronectin,
and

15 ii) a specific binding member for Extra Domain B (ED-B) of fibronectin isoform B (B-FN).

10. A kit according to claim 9 wherein said kit additionally comprises a specific binding member for a second isoform of
20 fibronectin.

11. A method of quantitating the amount of an insoluble marker protein in a sample of tissue or tumor, the method comprising:

25 homogenising a tissue or tumor sample to provide a homogenate;

contacting the homogenate with a specific binding member for the insoluble marker protein and incubating under conditions wherein the specific binding member binds the
30 insoluble marker protein if present in the homogenate;

separating insoluble material from soluble material in the homogenate, the soluble material including specific binding member unbound to the insoluble marker protein;

determining the amount of specific binding member in the insoluble material, correlating with the amount of insoluble marker protein in said sample.

5 12. A method according to claim 11 wherein the specific binding member is an antibody molecule that binds the insoluble marker protein.

13. A method according to claim 11 or claim 12 wherein the
10 insoluble marker protein is fibronectin isoform B (B-FN).

14. A method according to claim 13 wherein the specific binding member is specific to Extra Domain B (ED-B).

15 15. A method according to any one of claims 11 to 14 wherein the specific binding member is labelled with a radioisotope, an enzyme and/or a fluorescent moiety.

16. A method of determining grade or severity of neoplasia in
20 a sample of tissue or tumor, the method comprising:

determining the number of blood vessels in the sample;
determining the number of blood vessels in the sample
that contain fibronectin isoform B (B-FN);

25 calculating the fraction of blood vessels in the sample that contain B-FN to provide an angiogenic index (AI) of the tissue or tumor, wherein the angiogenic index is indicative of the grade or severity of neoplasia in the tissue or tumor.

17. A method according to claim 16, wherein the number of
30 blood vessels in the sample is determined by means of staining and/or by means of a specific binding member for a blood vessel marker.

18. A method according to claim 16 or claim 17 wherein the number of blood vessels that contain B-FN is determined using a specific binding member for B-FN.

5 19. A method according to claim 18 wherein the specific binding member for B-FN is specific to Extra Domain B (ED-B).

20. A method according to any one of claims 17 to 19 wherein the specific binding member for the blood vessel marker is an
10 antibody molecule.

21. A method according to any one of claims 18 to 20 wherein the specific binding member for B-FN is an antibody molecule.

15 22. A method according to any one of claims 17 to 21 wherein a specific binding member is labelled with a radioisotope, an enzyme and/or a fluorescent moiety.

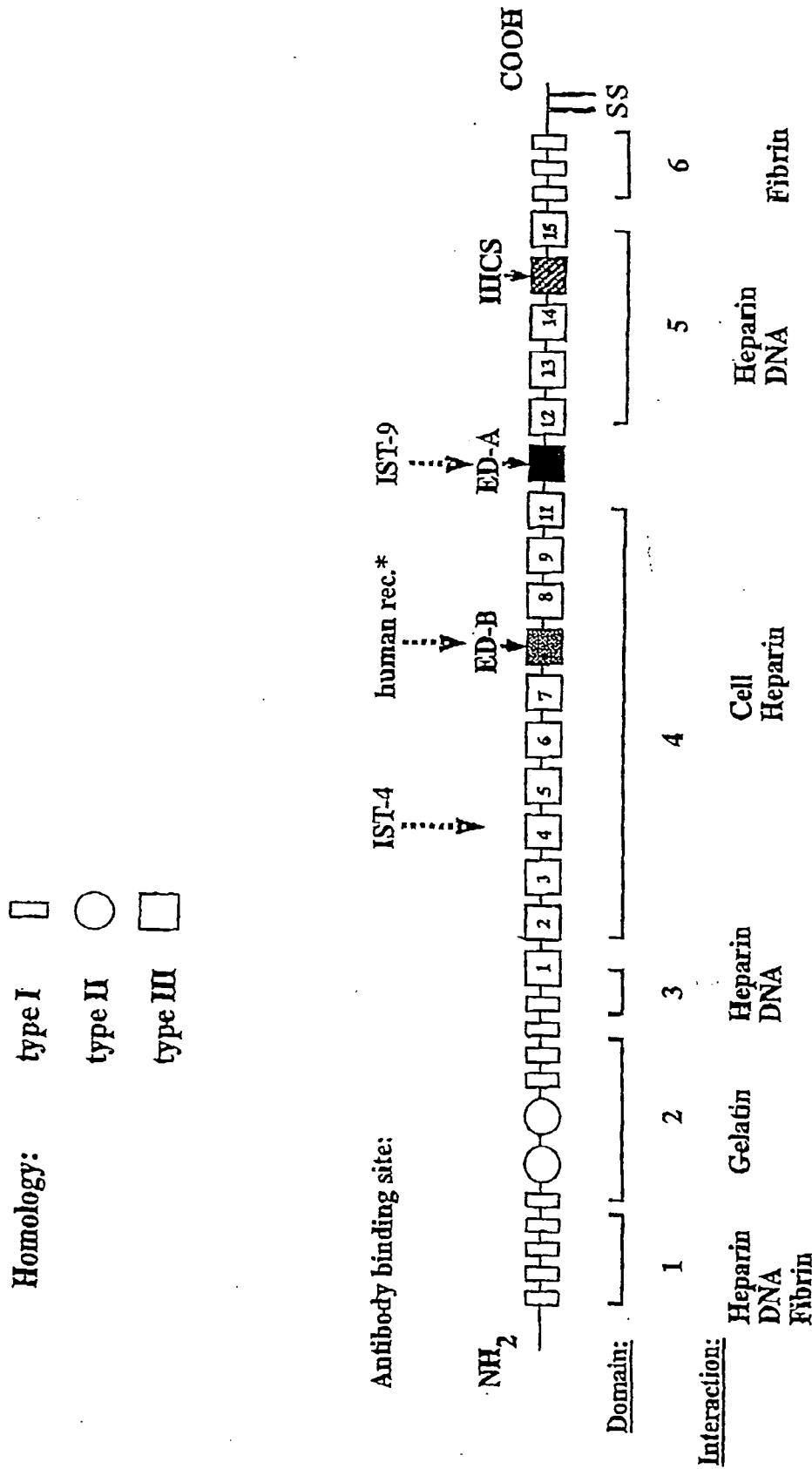


Fig. 1

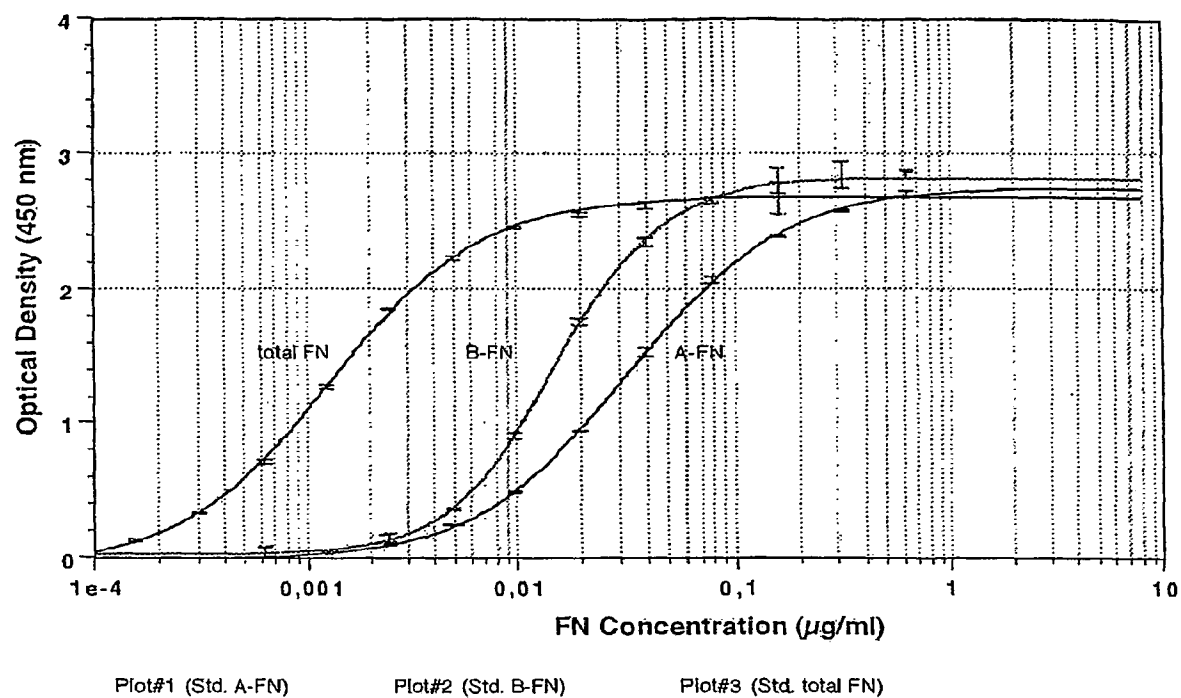


Fig. 2

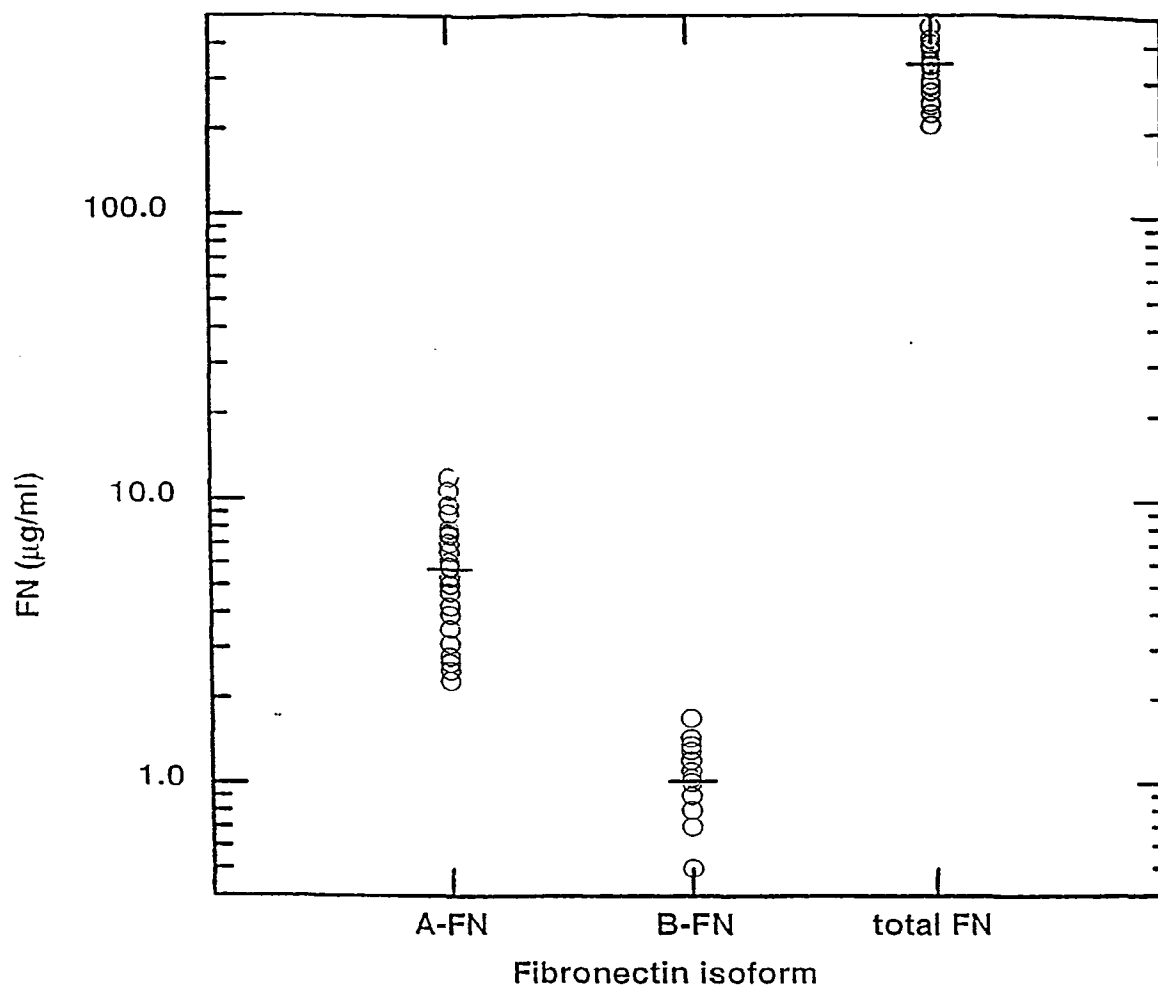


Fig. 3

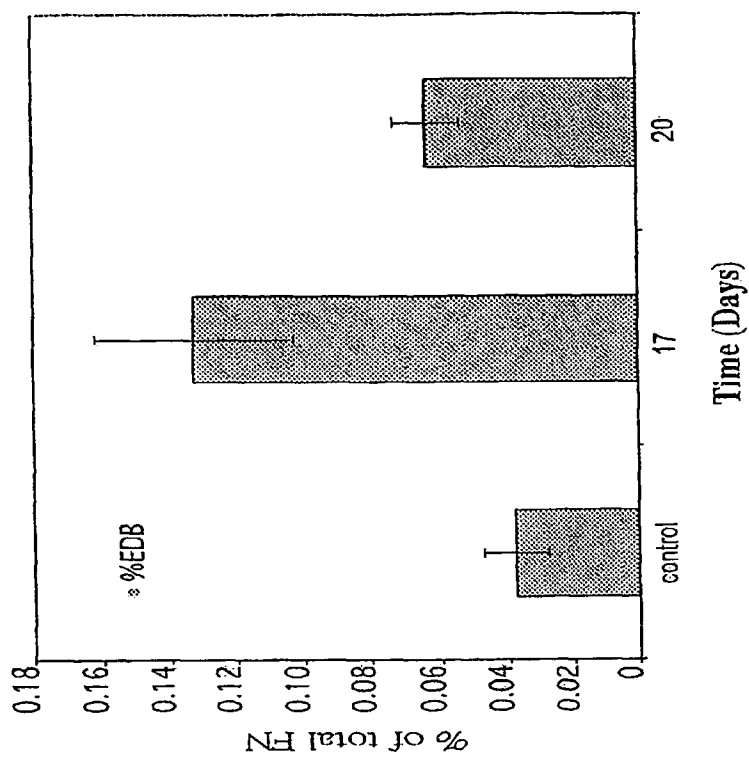


Figure 4B

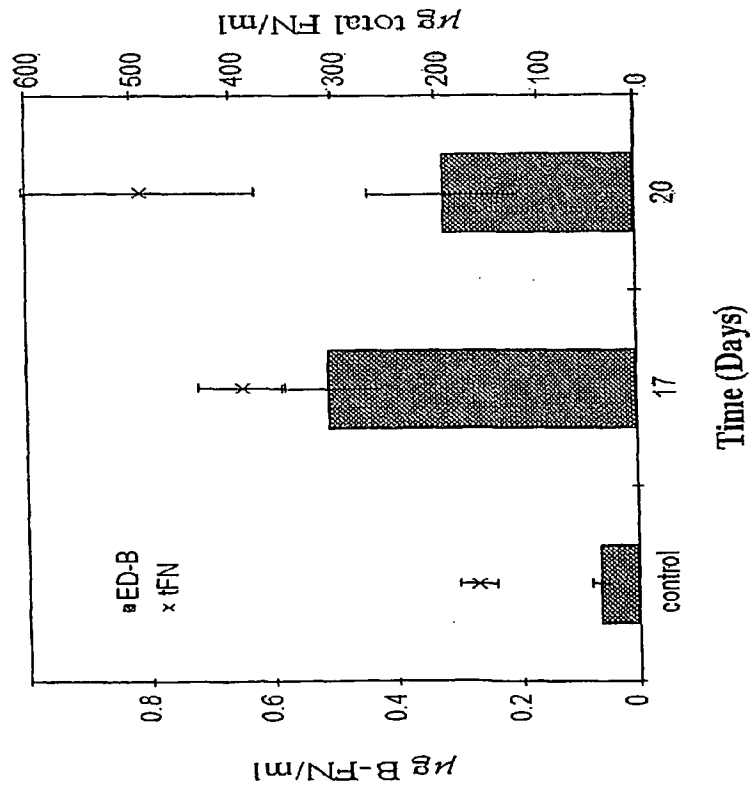


Figure 4A

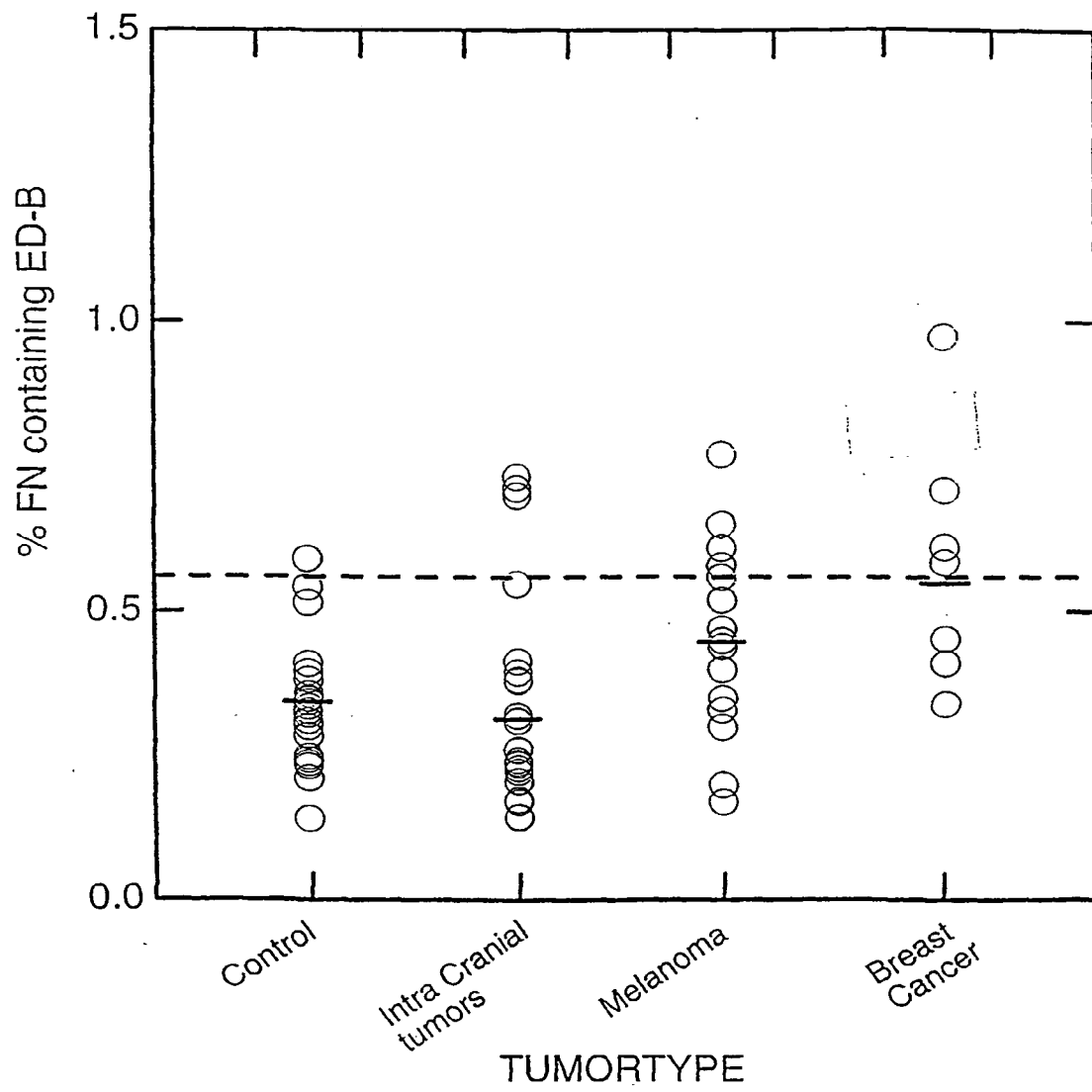


Fig. 5

6/11

Figure 6A

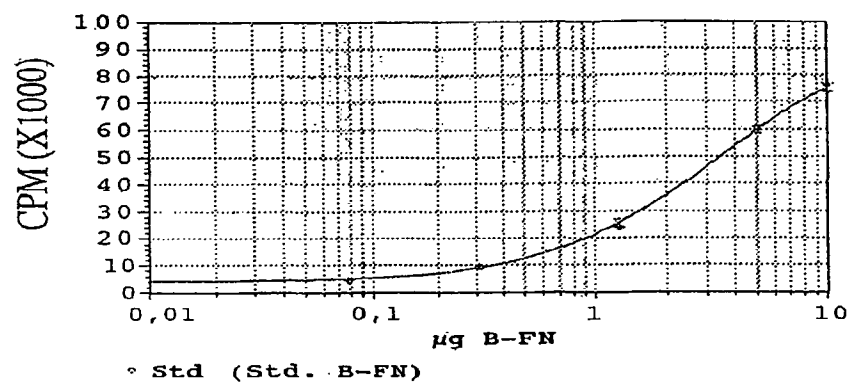
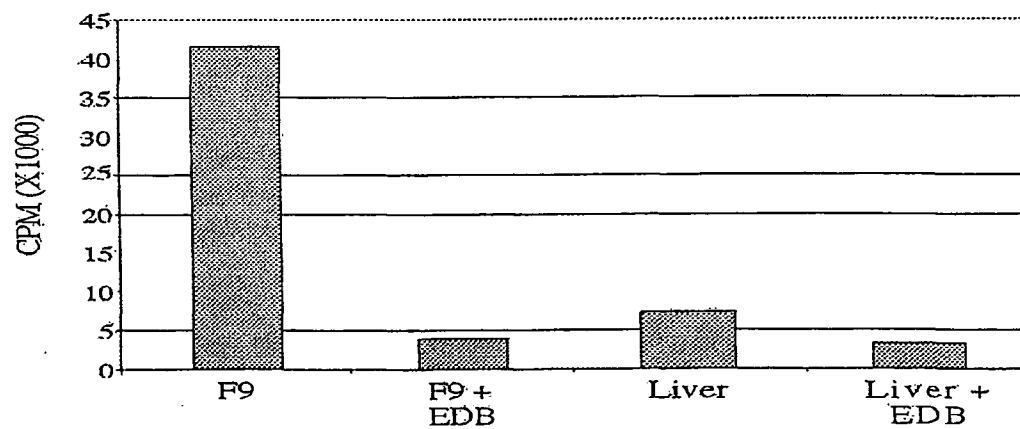


Figure 6B



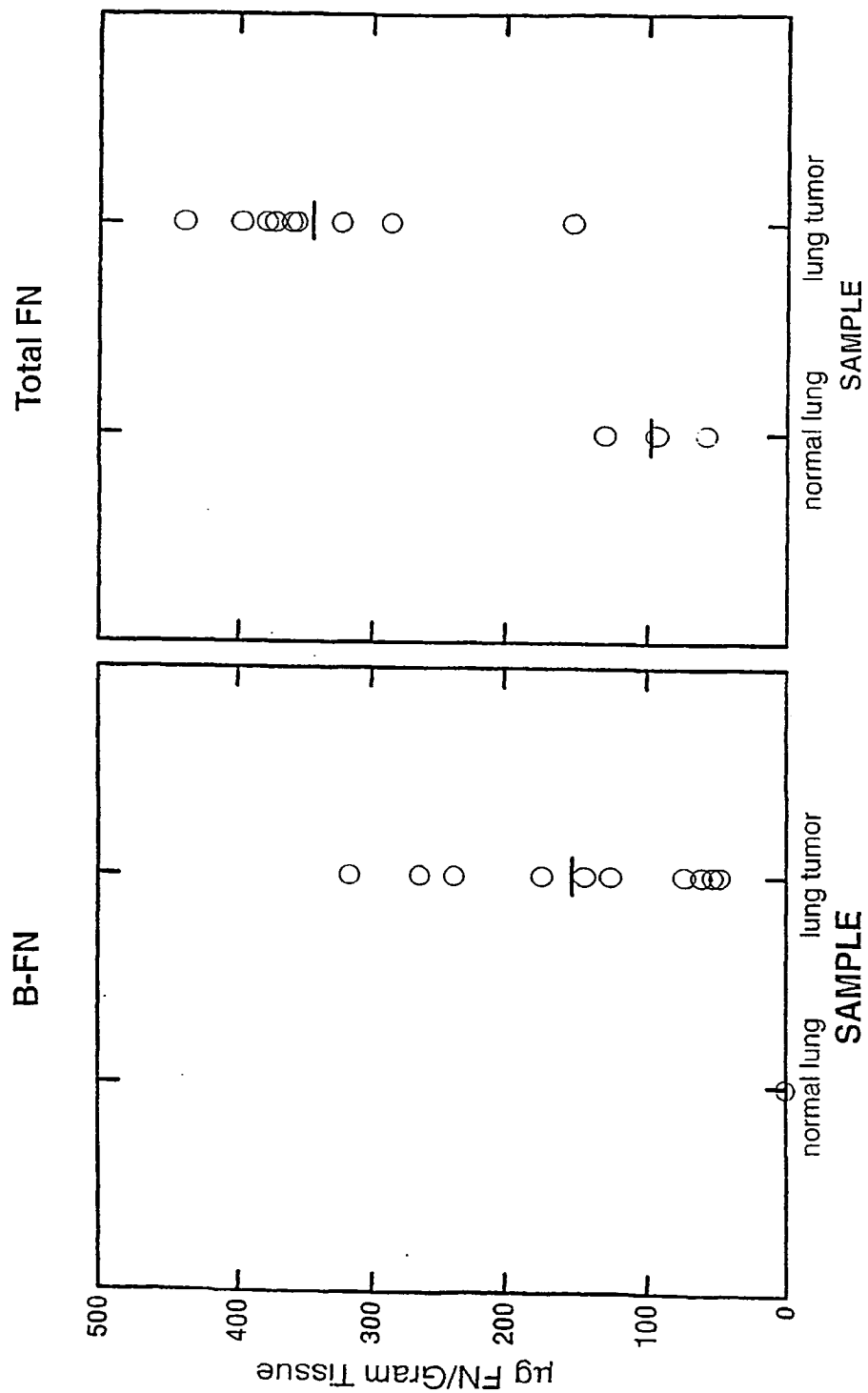


Fig. 7A

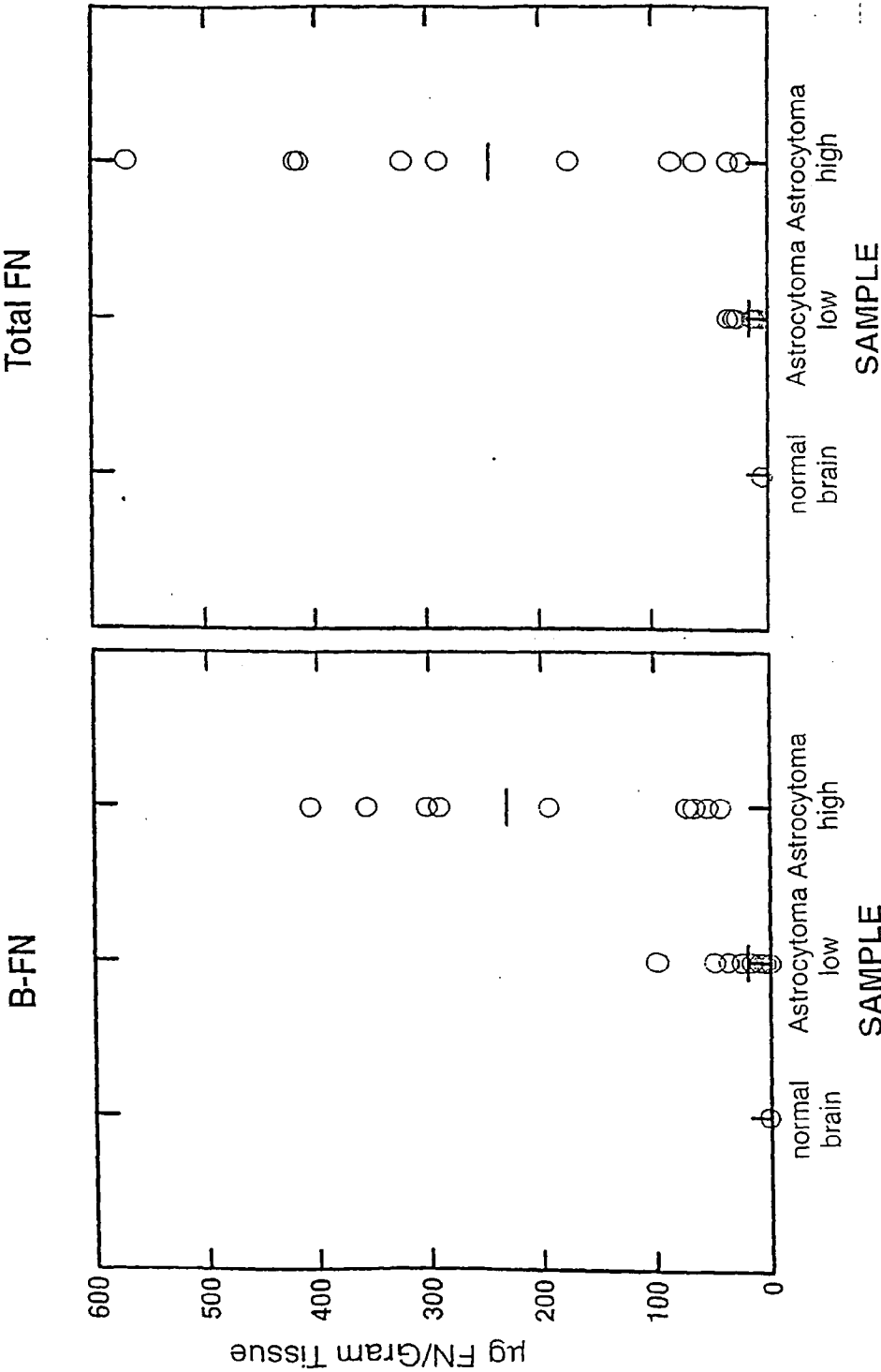


Fig. 7B

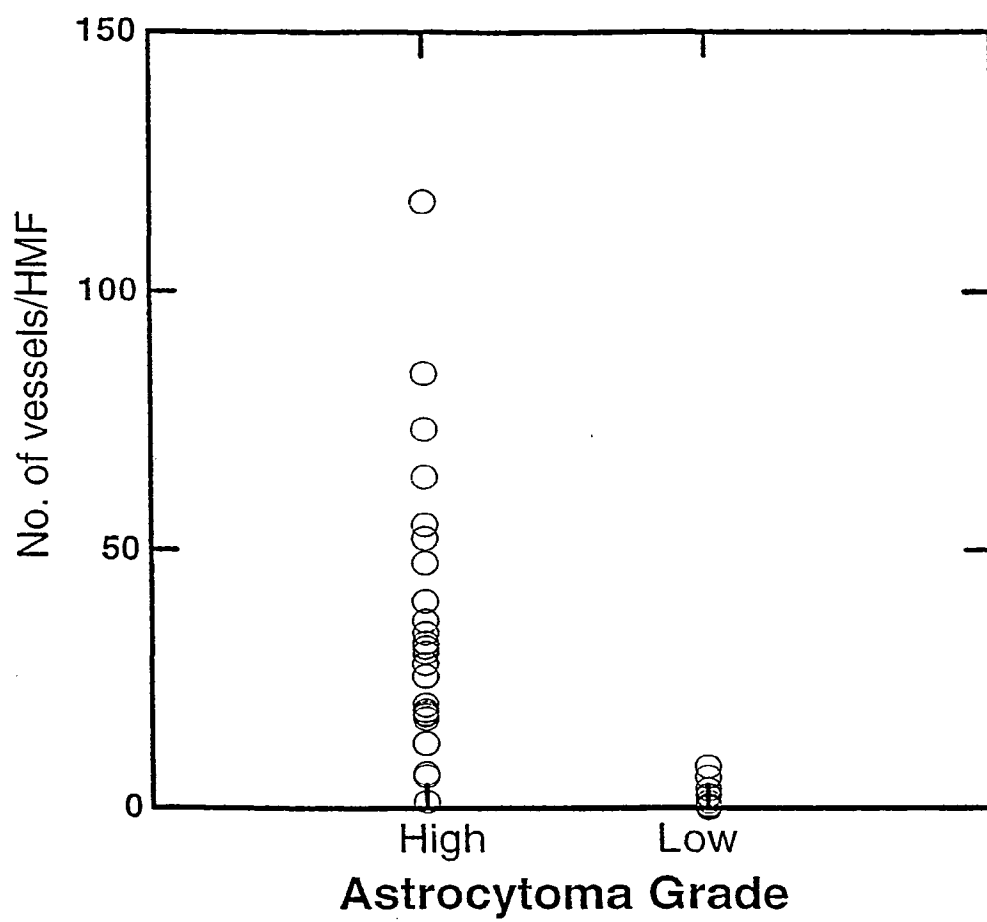


Fig. 8A

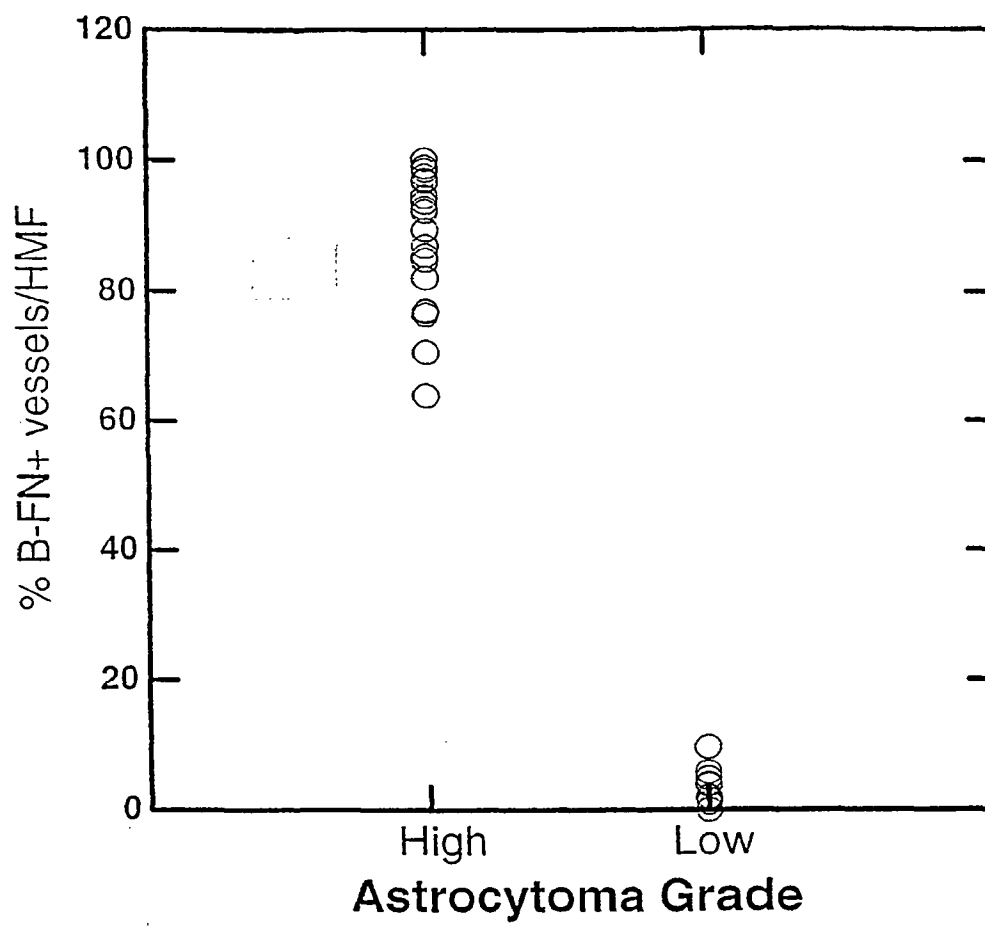


Fig. 8B

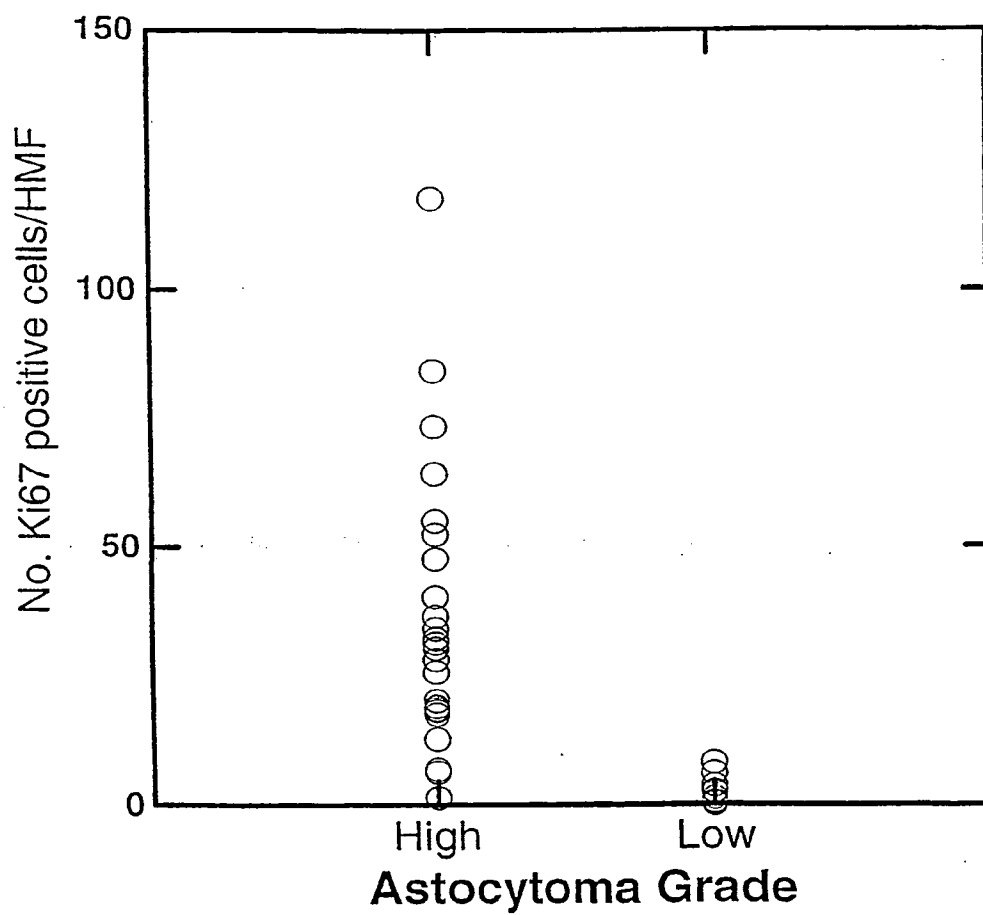


Fig. 8C

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **METHODS FOR QUANTITATIVE DETERMINATION OF B-FIBRONECTIN IN BIOLOGICAL FLUIDS AND TISSUES**

(57) Abstract: Quantitative determination of fibronectin isoform B (B-FN) and other insoluble marker proteins, including quantitative determination of B-FN in plasma and other biological fluids, employing gelatin that binds B-FN, also quantitative determination of insoluble marker proteins such as B-FN in tissue. Provision of quantitative information on angiogenesis through detection of B-FN as an indicator of malignancy in neoplasia.

WO 01/096599 A3

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/EP 01/06533

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CLAUDEPIERRE P ET AL.: "Increased Ed-B fibronectin plasma levels in spondyloarthropathies: comparison with rheumatoid arthritis patients and a healthy population." RHEUMATOLOGY, vol. 38, 1999, pages 1099-1103, XP002207493 cited in the application the whole document	1-10
Y	EP 0 355 882 A (DODDEMA HJ) 28 February 1990 (1990-02-28) the whole document	1-10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

6 December 2002

Date of mailing of the international search report

16/01/2003

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Fax: (+31-70) 340-3016

Authorized officer

Giry, M

INTERNATIONAL SEARCH REPORT

Interns Application No

PCT/EP 01/06533

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 45544 A (NERI D) 4 December 1997 (1997-12-04) page 10, line 12 - line 19 page 20, line 1 - line 5 page 14, line 1 - line 37 page 16, line 13 - line 18 ---	1-10
A	WO 90 08833 A (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 9 August 1990 (1990-08-09) page 37, line 18 -page 38, line 11 ---	1-10
X	WO 99 15647 A (DALY RJ ET AL.) 1 April 1999 (1999-04-01) page 4, line 1 -page 5, line 20 ---	11,12,15
Y	---	13,14
Y	CASTELLANI P. ET AL.: "The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis." INT. J. CANCER, vol. 59, 1 December 1994 (1994-12-01), pages 612-618, XP000960445 the whole document ---	13,14
X	D'OVIDIO MC ET AL.: "Intratumoral microvessel density and expression of ED-A/ED-B sequences of fibronectin in breast carcinoma." EUR. J. CANCER, vol. 34, no. 7, 1998, pages 1081-1085, XP004284989 the whole document -----	16-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 01/06533

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International Application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-10

Claims 1-10 relate to a method of determining the presence of fibronectin isoform B in a body fluid and kit therefor.

2. Claims: 11-15

Claims 11-15 relate to a method of quantitating the amount of an insoluble marker protein in a sample of tissue or tumor.

3. Claims: 16-22

Claims 16-22 relate to a method of determining grade or severity of neoplasia in a sample of tissue or tumor.

INTERNATIONAL SEARCH REPORT

ation on patent family members

Internat

Application No

PCT/EP 01/06533

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